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The Journal of Infectious Diseases

VOLUME 89, JULY-DECEMBER, 1951

TABLE OF CONTENTS

No. 1, July–August, 1951

PAGE

GRANGE S. COFFIN. Active immunization of birds against malaria 1

GRANGE S. COFFIN. Passive immunization of birds against malaria 8

ELERY R. BECKER, THOMAS M. SCHWINK, AND CHARLES E. BRODINE. The distribution of sparing and protective factors in plasma fractions of ducks recovered from lophurae malaria 16

HAROLD FRIEDLANDER. Effect of cortisone acetate on an experimental purulent infectious arthritis of white mice 26

L. A. STAUBER, H. A. WALKER, AND A. P. RICHARDSON. The in vitro agglutination of erythrocyte-free avian plasmodia 31

W. L. BARKSDALE AND AKIRA GHODA. Agglutinins for *Escherichia coli* in serum and in fecal extracts from human enteritides and from hyperimmunized and epinephrine-stimulated rabbits 35

W. L. BARKSDALE, AKIRA GHODA, AND KENZO OKABE. Coproagglutinins in ulcerative colitis 47

JEROME J. FRANKEL AND E. STATEN WYNNE. Antagonism by *Gaffkya tetragena* strains 52

H. S. KRAKAUER, R. RACHMAN, AND E. NETER. The effects of brucellergen skin test on brucella agglutinin titer in patients with brucellosis and other diseases 56

ARTHUR BROWN. The action of some water-soluble carcinogens on bacteria 59

ABRAHAM I. BRAUDE. Studies in the pathology and pathogenesis of experimental brucellosis. I. A comparison of the pathogenicity of *Brucella abortus*, *Brucella melitensis*, and *Brucella suis* for guinea pigs 76

ABRAHAM I. BRAUDE. Studies in the pathology and pathogenesis of experimental brucellosis. II. The formation of the hepatic granuloma and its evolution 87

ELERY R. BECKER, THOMAS M. SCHWINK, AND ROBERT M. PRATHER, JR. Hemagglutinin-inhibiting property of duck plasma exhibited in agglutination reactions involving duck erythrocytes and plasma of chicks recovered from lophurae malaria 95

ERWIN P. VOLLMER AND HERBERT S. HURLBUT. Ineffectiveness of cortisone therapy in mice infected with Japanese B encephalitis and the adverse effect of high doses 103

No. 2, September–October, 1951

JOSEPH S. INGRAHAM. Artificial radioactive antigens. I. Preparation and evaluation of S^{35}-sulfanilic acid-azo-bovine-γ-globulin 109

JOSEPH S. INGRAHAM. Artificial radioactive antigens. II. The metabolism of S^{35}-sulfanilic acid-azo-bovine-γ-globulin in normal and immune mice 114

AVIVAH ZUCKERMAN AND MEIR YOELI. The effect of splenectomy on the course of *Plasmodium berghei* infections in *Microtus guentheri* 130

WILLIAM H. TALIAFERRO AND LUCY G. TALIAFERRO. The role of the spleen in hemolysin production in rabbits receiving multiple antigen injections 143

STANLEY GREEN, MICHAEL G. WOHL, AND S. O. WAIFE. The effect of penicillin on typhoid antibody production 169

KENNETH M. HUGHES AND CLARENCE G. THOMPSON. A granulosis of the omnivorous looper, *Sabulodes caberata* Guenée 173

M. J. SURGALLA, J. L. KADAVY, M. S. BERGDOLL, AND G. M. DACK. Staphylococcal enterotoxin: production methods 180

F. MARILYN BOZEMAN, RAYMOND N. DOETSCH, AND JOHN E. FABER, JR. Some physiological properties of nonpathogenic mycobacteria 185

HELEN S. WELLS. Studies of the effect of antibiotics on infections with the mouse pinworm, *Aspicularis tetraptera*. I. The action of terramycin hydrochloride 190

J. P. USCAVAGE, MILAN NOVAK, AND W. I. TAYLOR. Prophylaxis of experimental diphtheria with penicillin 193

LA VERNE A. BARNES. Field trial of *Shigella flexneri III* vaccine. VII. Studies on asymptomatic carriers of the organism 199

No. 3, NOVEMBER–DECEMBER, 1951

NORMAN B. MCCULLOUGH AND C. WESLEY EISELE. Experimental human salmonellosis. III. Pathogenicity of strains of *Salmonella newport, Salmonella derby* and *Salmonella bareilly* obtained from spray-dried whole egg 209

LOIS M. JONES AND DAVID T. BERMAN. The pathogenicity of mucoid variants of brucellae for guinea pigs 214

SAMUEL SPECTOR AND ROBERT D. MERCER. The effect of pituitary adrenocorticotrophic hormone on experimental tetanus in mice 224

ERIC L. NELSON AND M. J. PICKETT. The recovery of L forms of brucella and their relation to brucella phage 226

WILLIAM BURROWS. Kinetic aspects of hemolysis by the α-toxin of *Clostridium welchii* and its inhibition by antitoxin 233

FLORENCE B. SEIBERT, CRETYL CRUMB AND EMMA H. DUFOUR. Antigenic differences in two tuberculin protein fractions 252

NORMAN B. MCCULLOUGH AND C. WESLEY EISELE. Experimental human salmonellosis. IV. Pathogenicity of strains of *Salmonella pullorum* obtained from spray-dried whole egg 259

NORMAN B. MCCULLOUGH AND GRACE A. BEAL. Growth and manometric studies on carbohydrate utilization of *Brucella* 266

ABRAHAM I. BRAUDE AND WESLEY W. SPINK. Studies in the pathology and pathogenes of experimental brucellosis. III. Investigations pertaining to the function of the spleen 272

ROBERT E. BOWLING AND E. STATEN WYNNE. Studies on the mechanism of antagonism by *Aerobacter* strains 277

LUIS M. GONZÁLEZ AND ENRIQUE KOPPISCH. Studies in bacillary dysentery. III. Mobilization of specific antibody into the intestine of the rabbit as a response to homologous living dysentery bacilli 281

WERNER BRAUN, ARTHUR N. GORELICK, MARY KRAFT AND DOROTHY D. MEAD. Studies on the in vivo fate of brucella variants with different virulence 286

MARGARET PITTMAN. Influence of sex of mice on histamine sensitivity and protection against *Hemophilus pertussis* 296

MARGARET PITTMAN. Comparison of the histamine-sensitizing property with the protective activity of pertussis vaccines for mice 300

J. Infect. Dis. Vol. 89, No. 3

EXPERIMENTAL HUMAN SALMONELLOSIS

III. PATHOGENICITY OF STRAINS OF *SALMONELLA NEWPORT*, *SALMONELLA DERBY*, AND *SALMONELLA BAREILLY* OBTAINED FROM SPRAY-DRIED WHOLE EGG

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In a previous paper, results of feeding three strains each of *Salmonella meleagridis* and *Salmonella anatum* derived from spray-dried whole egg to human volunteers were described.[1] The present paper deals with an extension of these studies to three other species, *Salmonella newport*, *Salmonella derby*, and *Salmonella bareilly*.

MATERIALS AND METHODS

Graduated dosages of the three species were fed to human volunteers and the subjects were closely studied thereafter. The materials and methods used in the entire study are presented in a previous paper[1] and will not be repeated in detail here. The strains of *S. newport*, *S. derby*, and *S. bareilly* used similarly had been isolated from market samples of spray-dried whole egg (4% to 6% moisture content) by the Bureau of Agricultural and Industrial Chemistry, U. S. Department of Agriculture.

RESULTS

All cases of illness produced by feeding the three species are described in detail in the section on Description of Clinical Cases.

The results of feeding *S. newport* at three dosage levels are presented in table 1. With the lowest level fed, 152,000 organisms, one man in a group of 6 became ill; with the second level, 385,000 organisms, one man in a group of 8 became ill; with the highest level, 1,350,000 organisms, half of the 6 subjects developed illness. The agglutination titer rose two dilutions or more in two of the 5 individuals becoming ill and remained unchanged in the other three. The organism was recovered from the stools of 15 of the 20 men fed; in 7 subjects positive stool cultures persisted for 14 days or longer and in one of the clinical cases for 51 days.

S. derby was fed at 5 dosage levels (table 2) varying from 138,500 to 15,000,000 organisms. No illness resulted until the highest dosage level was fed, when three of the 6 subjects became ill. The agglutination titer rose in two of these men but did not change in the third. The organism was recovered from the stool after 18 of the 30 feedings but in general tended to disappear quickly from the stool. However, two individuals not becoming ill carried the organism for 36 and 44 days.

S. bareilly was fed to groups of 6 men each at three dosage levels: 125,000; 695,000; and 1,700,000 organisms. The results are presented in table 3. Illness developed in one man at the lowest level, in two men at the second level,

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TABLE 1.—*Experimental feeding of* S. newport *to human volunteers.*

Feeding no.	No. of previous feedings	No. of organisms fed	Aggl. titer		Fecal cultures	
			Initial	Maximal	No. of times isolated	Day last recovered
234	0	152,000	1:20	1:20	0	0
235	0	152,000	1:20	1:20	4	9
236	0	152,000	Neg.	Neg.	0	0
*237	0	152,000	1:20	1:160	7	51
238	0	152,000	Neg.	1:20	3	14
239	0	152,000	Neg.	1:20	0	0
*240	1	385,000	1:20	1:20	1	2
241	1	385,000	1:20	1:20	2	4
242	1	385,000	1:20	1:20	0	0
243	1	385,000	1:40	1:40	0	0
244	0	385,000	1:20	1:40	8	28
245	0	385,000	1:20	1:20	2	4
246	0	385,000	1:20	1:20	5	11
247	0	385,000	1:80	1:80	6	14
248	2	1,350,000	1:40	1:40	2	7
*249	2	1,350,000	Neg.	Neg.	5	9
*250	1	1,350,000	Neg.	Neg.	7	16
251	0	1,350,000	Neg.	Neg.	7	16
*252	0	1,350,000	Neg.	1:40	7	16
253	0	1,350,000	Neg.	Neg.	3	7

* Clinical illness described under section on DESCRIPTION OF CLINICAL CASES.

and in 4 of the 6 men fed at the highest level. The agglutination titer rose two dilutions or more in 4 of the 7 subjects who became ill. The organism was recovered from the stool after 16 of the 18 feedings but disappeared promptly.

In all of the experiments, in subjects not listed as developing illness, there were no symptoms or signs referable to the feedings, nor was there a significant change in agglutination titer in any instance.

In 11 of the 15 clinical cases of illness described, the incubation period ranged

TABLE 2.—*Experimental feeding of* S. derby *to human volunteers.*

Feeding no.	No. of previous feeding	No. of organisms fed	Aggl. titer		Fecal cultures	
			Initial	Maximal	No. of times isolated	Day last recovered
254	0	138,500	Neg.	Neg.	2	3
255	0	138,500	1:20	1:20	0	0
256	0	138,500	Neg.	Neg.	5	36
257	0	138,500	1:20	1:20	0	0
258	0	138,500	1:20	1:20	0	0
259	0	138,500	Neg.	Neg.	1	1
260	1	705,000	Neg.	Neg.	1	2
261	1	705,000	1:20	1:40	1	2
262	1	705,000	Neg.	Neg.	0	0
263	0	705,000	1:40	1:40	1	2
264	0	705,000	Neg.	Neg.	2	11
265	0	705,000	1:20	1:20	0	0
266	1	1,655,000	1:40	1:40	1	2
267	1	1,655,000	Neg.	Neg.	0	0
268	0	1,655,000	Neg.	Neg.	4	9
269	0	1,655,000	Neg.	Neg.	2	4
270	0	1,655,000	Neg.	Neg.	1	2
271	0	1,655,000	Neg.	Neg.	0	0
272	2	6,400,000	Neg.	Neg.	1	2
273	1	6,400,000	1:40	1:40	0	0
274	1	6,400,000	Neg.	Neg.	3	7
275	0	6,400,000	1:20	1:40	7	44
276	0	6,400,000	Neg.	Neg.	0	0
277	0	6,400,000	Neg.	Neg.	0	0
*278	1	15,000,000	1:20	1:80	5	11
279	1	15,000,000	Neg.	Neg.	1	2
280	1	15,000,000	Neg.	Neg.	0	0
281	1	15,000,000	Neg.	Neg.	0	0
*282	0	15,000,000	1:80	1:80	2	4
*283	0	15,000,000	Neg.	1:80	5	11

* Clinical illness described under section on DESCRIPTION OF CLINICAL CASES.

from 27 to 48 hours. Two cases (*S. derby*) had short incubation periods of 4 and 5 hours, and one (*S. newport*) an incubation period of 7 days.

As in the previously reported feeding experiments, some feeding groups contained individuals who had previously been fed lower dosages of the same organism without production of illness or the development of a carrier state. No difference could be observed in the occurrence of experimental illness between these subjects and those having no previous experimental experience with the organism. This is in agreement with the previously reported experiments with *S. meleagridis* and *S. anatum*.[1]

TABLE 3.—*Experimental feeding of* S. bareilly *to human volunteers.*

Feeding no.	No. of previous feedings	No. of organisms fed	Aggl. titer		Fecal cultures	
			Initial	Maximal	No. of times isolated	Day last recovered
284	0	125,000	Neg.	Neg.	1	1
285	0	125,000	Neg.	Neg.	1	1
286	0	125,000	1:20	1:20	0	0
287	0	125,000	Neg.	Neg.	1	1
*288	0	125,000	Neg.	1:80	4	6
289	0	125,000	1:40	1:80	2	3
*280	1	695,000	Neg.	Neg.	1	4
291	1	695,000	1:80	1:80	3	7
292	1	695,000	Neg.	Neg.	1	2
293	0	695,000	1:40	1:40	1	2
294	0	695,000	Neg.	Neg.	3	9
*295	0	695,000	Neg.	1:80	5	11
*296	2	1,700,000	Neg.	1:40	1	2
*297	1	1,700,000	Neg.	Neg.	3	7
298	1	1,700,000	Neg.	Neg.	0	0
*299	0	1,700,000	Neg.	1:40	2	3
*300	0	1,700,000	Neg.	Neg.	2	3
301	0	1,700,000	Neg.	Neg.	1	2

* Clinical illness described under section on DESCRIPTION OF CLINICAL CASES.

DESCRIPTION OF CLINICAL CASES

Salmonella newport

No. 237 received 152,000 organisms of *S. newport*. Seven days later abdominal cramping, nausea, and diarrhea commenced. The highest recorded temperature during the first day of illness was 99.4 F; readings thereafter were normal. There were 8 loose watery stools the first day and approximately 25 the second day. Abdominal cramping and tenderness accompanied by mild nausea persisted for three days. The agglutination titer rose from 1:20 to 1:160. The organism was last recovered from the stool 51 days after feeding.

No. 240 received 385,000 organisms of *S. newport*. Twenty-seven hours later there was a sudden onset of nausea and vomiting which persisted for several hours, and he was hospitalized. The temperature was normal and remained so throughout hospitalization. The white blood count was 12,800. He complained of abdominal cramps and headache. On the second day of illness there were three loose watery stools. There was no change in the agglutination titer. The organism was recovered from the stool on one occasion only, two days after feeding.

No. 249 received 1,350,000 organisms of *S. newport*. Twenty-eight hours later abdominal cramping, nausea, and diarrhea commenced. He had two chills during the night. The temperature was not obtained until the next morning and was then 99.8 F and the white blood count was 8000 with 69% polymorphonuclears. There were 6 loose watery stools during the first day of illness and three the next day. His abdomen was moderately tender upon palpation, and he complained of abdominal cramping, headache, and generalized aching. There was low grade fever for two days. A blood culture remained sterile. The agglutination test remained negative. The organism was last recovered from the stool 9 days after feeding.

No. 250 received 1,350,000 organisms of *S. newport*. Forty-eight hours later he was asymptomatic, but his temperature was 99.4 F. Fifty-two

1. McCullough, N. B. and Eisele, C. W. 1951, J. Infect. Dis. **88**: 278–289.

hours after feeding diarrhea commenced. There were 5 or 6 loose watery stools per day for three days. He had no other complaints. There was low grade fever of 99.2 to 100 F for 7 days. The agglutination test remained negative. The organism was last recovered from the stool 16 days after feeding.

No. 252 received 1,350,000 organisms of *S. newport*. Forty-eight hours later he complained of general malaise, nausea, a feverish feeling, and occasional abdominal cramps. His bowels had not moved since the feeding. He vomited in the examining room. His temperature was 101 F and the white blood count was 8000 with 63% polymorphonuclears. He was admitted to the hospital. Fever continued for 4 days with a peak of 101.8 F. He had profuse sweats and complained of repeated chills, headache, and backache. There were two loose stools on the second day of hospitalization, otherwise bowel function was normal. Convalescence was complete 7 days after the onset. Two blood cultures were negative. The agglutination titer rose from negative to 1:40. The organism was last recovered from the stool 16 days after feeding.

Salmonella derby

No. 268 received 15,000,000 organisms of *S. derby*. Thirty-one hours later diarrhea commenced with 6 loose watery stools during the next 12 hours. He complained of nausea, abdominal rumbling, and excessive flatus. These complaints continued, and there were occasional watery stools for the next 7 days. All recorded temperature readings were normal. The agglutination titer rose from 1:20 to 1:80. The organism was last recovered from the stool 11 days after feeding.

No. 282 received 15,000,000 organisms of *S. derby*. Five hours later diarrhea commenced and persisted for three days accompanied by mild nausea for the first two days. There were three loose watery stools in the first 6 hours of illness and two or three per day thereafter. The temperature was not obtained the first evening of illness, and all the recorded readings thereafter were normal. There was no change in the agglutination titer. The organism was last recovered from the stool 4 days after feeding.

No. 283 received 15,000,000 organisms of *S. derby*. Four hours later abdominal cramping, nausea, and diarrhea commenced. He refused dinner because of nausea. There were 5 loose watery stools in the first 12 hours of illness and three per day for two additional days. Abdominal cramping and nausea persisted during this period. There was low grade fever for 7 days, the temperature rising to 99.4 and 99.6 F. The white blood count on the second day of illness was 6000. The agglutination titer rose from negative to 1:80. The organism was last recovered from the stool 11 days after feeding.

Salmonella bareilly

No. 288 received 125,000 organisms of *S. bareilly*. Twenty-eight hours later abdominal cramping and diarrhea commenced. He complained of alternate chilling and flushing and almost continuous diarrhea all night. He was admitted to the hospital the next morning, at which time the temperature was 99.6 F and the white blood count was 5000. During the first 24 hours in the hospital there were 18 loose watery stools, the following day 12, and 7 or 8 per day for the next three days. He vomited on two occasions and complained of severe headache, general malaise, abdominal cramping and tenderness. There was no fever after the first hospital day. He was released from the hospital on the 8th day after the onset of illness. Two blood cultures remained sterile. The agglutination titer rose from negative to 1:80. The organism was last recovered from the stool 6 days after feeding.

No. 290 received 695,000 organisms of *S. bareilly*. Sixty-six hours later abdominal cramping and diarrhea commenced. There were 5 loose watery stools during the next 24 hours and three or four per day on the two succeeding days. The recorded temperature readings were all normal. The agglutination test remained negative. The organism was last recovered from the stool 4 days after feeding.

No. 295 received 695,000 organisms of *S. bareilly*. Commencing on the second day thereafter and persisting for 5 days, he complained of having two or three loose stools per day. He offered no other complaints. On one occasion a temperature reading of 99.4 F was obtained. The agglutination titer rose from negative to 1:80. The organism was last recovered from the stool 11 days after feeding.

No. 296 received 1,700,000 organisms of *S. bareilly*. Forty-two hours later abdominal cramping and diarrhea commenced. There were 4 loose watery stools in the next 6 hours. The temperature at that time was 99 F, and subsequent readings were all normal. Diarrhea continued for three days with 4 to 7 loose watery stools per day. He complained of headache, a flushed feeling, abdominal cramps and nausea. The agglutination titer rose from negative to 1:40. The organism was recovered from the stool on one occasion only, two days after feeding.

No. 297 received 1,700,000 organisms of *S.*

bareilly. Forty hours later diarrhea commenced. There were 4 loose watery stools in the next 6 hours. The temperature at that time was 99.2 F, and the white blood count was 8500. He complained of abdominal rumbling, excessive flatus, and general malaise. The following day he was asymptomatic. The agglutination test remained negative. The organism was last recovered from the stool 7 days after feeding.

No. 299 received 1,700,000 organisms of *S. bareilly*. Forty-eight hours later he was asymptomatic, but his temperature was 99.4 F. Diarrhea commenced 56 hours after feeding. There were 5 loose watery stools in the next 12 hours and three additional stools the next day. Low grade fever was present for three days, and he complained of aching of his legs. The agglutination titer rose from negative to 1:40. The organism was last recovered from the stool three days after feeding.

No. 300 received 1,700,000 organisms of *S. bareilly*. Thirty-six hours later diarrhea commenced. There were 4 loose watery stools in the next 12 hours but no other complaints. His temperature at that time was 102.2 F, and the white blood count was 7500 with 70% polymorphonuclears. He was admitted to the hospital. He complained of abdominal cramping and soreness and had profuse sweats during the night. There were several loose stools during the first two days in the hospital. The temperature became normal 18 hours after admission. A blood culture remained sterile. The agglutination test remained negative. The organism was last recovered from the stool three days after feeding.

DISCUSSION

As with the previous experiments,[1,2] the presently reported work was done in a state prison. The advantages of such a closed environment for work of this nature have been discussed. Likewise, critical selection of volunteers, the use of adequate control feedings, and the necessity of objective criteria of illness were emphasized. Here again, although the subjects were unrestricted unless hospitalized for illness, no evidence of person to person spread of infection was found.

The small dosages of *S. bareilly* and of *S. newport* producing illness are worthy of note. One man became ill at the lowest dosage level of each organism, 125,000 and 152,000 respectively. With *S. bareilly*, the illness was severe enough to require hospitalization for 8 days.

As in the case of the previously reported illnesses due to *S. meleagridis* and *S. anatum*,[1,2] there was considerable variation in the manifestations, severity, and duration of illness. The range was from a mild brief diarrhea to severe gastroenteritis accompanied by diffuse systemic complaints.

Likewise the incubation period was variable. Although in the majority of cases it was in the range usually accredited to salmonellosis, there were two instances (cases 283 and 282) of unusually short incubation periods of 4 and 5 hours. These two are within the limits associated with staphylococcus enterotoxin food poisoning. That one case (no. 237) presenting a typical illness had an incubation period of 7 days is also noteworthy.

SUMMARY

Experimental human salmonellosis was produced by feeding volunteers strains of *Salmonella newport*, *Salmonella derby*, and *Salmonella bareilly* isolated from spray-dried eggs. Graduated dosages of each species were fed. With dosages of 1,350,000; 15,000,000; and 1,700,000 organisms, respectively, 50% or more of the subjects became ill. With both *S. bareilly* and *S. newport* one subject became ill at the lowest dosage level fed, 125,000 and 152,000 organisms, respectively. Descriptions of the clinical illness in 15 subjects are presented.

2. McCullough, N. B. and Eisele, C. W. 1951, J. Immunol. **66**: 595–608.

THE PATHOGENICITY OF MUCOID VARIANTS OF BRUCELLAE FOR GUINEA PIGS

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The disease produced by parenteral inoculation of brucellae into guinea pigs is usually chronic, retrogressive and rarely fatal. The degree of involvement of the reticulo-endothelial system may be taken as a measurement of the pathogenicity of a culture and may be evaluated, in part, by observation of the gross lesions and the persistence of the organisms in the animal tissues. Although few comprehensive studies have been reported, most workers have considered nonsmooth variants of brucellae to be of relatively low pathogenicity.

Henry (1933) found that rough cultures were capable of general distribution in the guinea pig early in infection but incapable of persisting beyond the 5th week. Other workers (Zdrodowski et al, 1930; Marshall and Jared, 1931) reported that a few nonsmooth variant cultures could be recovered from guinea pig tissues as long as 7 and 8 weeks after inoculation, but the majority of cultures studied were eliminated in the first few weeks. Huddleson (1947) recovered mucoid organisms from guinea pig tissues at 3 weeks but not at 4 weeks, or later, after intraperitoneal or subcutaneous inoculation.

The intermediate variant, *Brucella abortus* strain 45/20 (McEwen and Priestley, 1938), persisted in guinea pigs for at least 13 weeks after inoculation (McEwen, 1940). When inoculated into pregnant cattle, strain 45/20 caused abortion, and smooth virulent *Brucella* could be recovered from the animals. Edwards et al (1945) examined the cultural and serological characteristics of *Brucella* recovered from nonpregnant cattle which had been vaccinated with strain 45/20 and sacrificed at various time intervals following vaccination. The cultures displayed combinations of antigenicity, virulence, and carbon dioxide requirement which differed from those of the parent strain 45/20, thus demonstrating the ability of these characteristics to vary independently of each other.

Braun and Hauge (1948) were unable to recover nonsmooth variants from guinea pigs and rabbits 9 and 10 weeks after inoculation of rough, intermediate and mucoid cultures, but did isolate such variants from mice 5 weeks after inoculation. The presence of a "smooth selective" factor in the serum of guinea pigs and rabbits was correlated with host resistance to the variant culture. During the course of infection with a smooth strain of *Brucella suis* in guinea pigs Braun (1950a) reported that the serum selective factor disappeared, which permitted nonsmooth types to become established in the animal.

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The implications of Braun's studies on the serum selective factor as a part of the mechanism of resistance to *Brucella* infections, and the current interest in vaccines for cattle produced from mucoid cultures prompted this investigation of the pathogenic potentialities of mucoid *Brucella*, alone, and in mixed infections with smooth *Brucella* which were inoculated previously to and simultaneously with the mucoid culture.

MATERIALS AND METHODS

Stock cultures were maintained on tryptose agar slants at 4 C. The 2% glycerine, 1% dextrose agar (2-1 agar) recommended by Henry (1933) was used for all plate cultures in order that examination of colonial morphology might be made. The plates were incubated at 37 C for 4 days, and the colonies were observed at a magnification of 12 diameters with the aid of a stereoscopic microscope, using obliquely transmitted light. Morphological observations were supplemented by the acriflavine test (Braun and Bonestell, 1947), which is reported to be a reliable indication of the antigenic activity of the variant in vivo (Braun and Hauge, 1948). Streptomycin resistant strains were cultivated on 2-1 agar containing one mg streptomycin (calcium chloride complex) per ml.

Male guinea pigs weighing between 250 and 500 g were inoculated intraperitoneally with 0.5 or 1.0 ml of the bacterial suspensions in 1% tryptone water. Viable cell counts and examinations of colonial morphology were made of all inoculums by plating on 2-1 agar. Representative animals from each group were sacrificed at successive weekly intervals following inoculation. Blood was withdrawn by cardiac puncture, and the animals were examined for gross pathological changes. The regional lymph nodes, spleen, liver, and testes from each animal were minced and streaked on 2-1 agar plates both with and without streptomycin to determine the presence or absence of *Brucella* in each of the organs.

The serums from all animals were tested for the presence of agglutinins for both smooth and mucoid antigens by the test tube method. The mucoid antigen was prepared by suspending the organisms of the particular culture being investigated in 0.5% phenolized saline, and adjusting to the approximate turbidity of the standard tube antigen supplied by the Bureau of Animal Industry. Suspensions of both the mucoid and smooth antigens were stable under the conditions of the test.

Sources of strains employed

Br. abortus strain 2308 was obtained originally from the Bureau of Animal Industry. This is a virulent strain which does not require added carbon dioxide for growth. The smooth culture (2308S) used in this study was isolated from an experimentally infected guinea pig in this laboratory. The mucoid variant (2308M) was selected as a single colony following cultivation of the smooth culture on 2-1 agar.

Two *Br. suis* mucoid variants (*suis* M and *suis* R^M) were supplied by Dr. Werner Braun. The R^M culture was so designated because its colonial appearance was rough, but it was agglutinated like mucoid organisms in the acriflavine test. A third *Br. suis* mucoid culture was obtained by picking a single colony from a plating of mucoid vaccine supplied by Dr. I. F. Huddleson. These colonies were bluish gray on 2-1 agar and were mucoid to the acriflavine test.

Br. abortus strain 6980 and *Brucella melitensis* strain 56741 were recovered from human blood by the Wisconsin State Laboratory of Hygiene. Mucoid variants were selected after 10 days growth of the originally smooth cultures in buffered broth. *Br. abortus* strain 6980 did not require added carbon dioxide for growth.

Streptomycin resistance (SMR) was employed as a genetic marker on the mucoid cultures studied. This resistance persisted as a characteristic of the culture in the absence of the drug, after animal passage, and after reversion from mucoid to smooth colonial morphology. The pathogenicity of the cultures for guinea pigs was apparently unaltered by the drug resistant characteristic.

Cultures resistant to 1 mg streptomycin per ml were obtained at an average frequency of three resistant colonies per 10^{10} organisms inoculated on 2-1 agar containing 1 mg streptomycin per ml. The mutation rate to streptomycin resistance could not be determined (at least under these conditions) inasmuch as nonresistant organisms were able to divide several times in the presence of streptomycin concentrations, which eventually rendered them nonviable (Jones, 1950).

The use of streptomycin resistant cultures offered several advantages in infection experiments. Recoveries of organisms from animal tissues could be made on mediums containing streptomycin, thus eliminating interference by bacterial contaminants. In mixed infections with streptomycin resistant mucoid and streptomycin sus-

ceptible smooth cultures, the resistant organisms could be selected on streptomycin mediums even when present in numbers so small that they could not be detected in the presence of large numbers of smooth susceptible colonies growing on the streptomycin free medium.

RESULTS

Infection with Br. abortus *strain SMR2308M*

The summary of the data pertaining to the persistence of *Br. abortus* strain SMR2308M in 54 guinea pigs is presented in table 1. Nine weeks was set as an arbitrary limit for observation in these experiments. An inoculum of 1.25×10^6 mucoid organisms established an infection which was maintained in some, but not all, of the animals for as long as 9 weeks. Inoculation with fewer than 10^4 organisms resulted in elimination of the infection in the first few weeks. However, only a few animals were involved in what might have represented the threshold area.

Slight enlargement of the lymph nodes and spleen and abscess formation in lymph nodes, spleen, and epididymis of some animals injected with 10^6 or more organisms were evident during the third and fourth weeks of infection. Regression apparently occurred inasmuch as the organs of animals sacrificed the 5th week and later appeared grossly normal.

Only smooth organisms, most of which were streptomycin resistant, were recovered from two guinea pigs which had been inoculated with 10^8 organisms of SMR2308M and sacrificed the 7th and 8th weeks after inoculation. These animals displayed lesions typical of infection with a virulent culture of *Br. abortus*. The smooth organisms apparently originated from the mucoid inoculum by mutation, proliferated, and became established in the animal tissues, whereas the mucoid forms were entirely eliminated.

Agglutinins for the mucoid antigen were not demonstrable in any of the serums. Only serums of animals from which smooth organisms were recovered agglutinated the smooth antigen.

TABLE 1.—*Persistence of* Br. abortus *strain SMR2308M in guinea pigs.*

Number of organisms injected	Weeks after inoculation									Totals
	1	2	3	4	5	6	7	8	9	
75	0/3*	0/3								0/6
7.5×10^3	0/1	0/5								0/6
7.5×10^5		0/2	0/2							0/4
7.5×10^4			1/4							1/4
1.25×10^6			3/3	2/3	1/3	1/3	0/3	0/3	1/2	8/20
3×10^8			1/1	1/1	1/1	1/1	1/1	1/1	0/1	6/7
3×10^8			1/1	1/1	1/1	1/1	1/1†	1/1†	1/1	7/7
Totals										22/54

* Number infected/number examined.
† These animals yielded pure cultures of smooth *Brucella*.

Mixed infections of mucoid and smooth organisms

The apparent inability of mucoid organisms to maintain themselves in the presence of the smooth form in the guinea pig, indicated in the results just described, prompted experiments with mixed infections. Twenty-one guinea pigs were inoculated simultaneously with 10^6 organisms of SMR2308M and 10^3 organisms of 2308S. The results are included in table 2.

Both mucoid and smooth organisms were recovered from the lymph nodes of animal 2, sacrificed three weeks after inoculation, whereas the spleen of this guinea pig yielded large numbers of

smooth *Brucella* only. All organisms isolated from the spleen and lymph nodes of animal 3, killed at the same time, were mucoid. Some mucoid organisms were recovered from the lymph nodes of two guinea pigs of the three sacrificed at 5 weeks, although the greater proportion of colonies from these only 5 of the 21 guinea pigs.

Agglutinins for the smooth antigen were demonstrated only in the serums from animals infected with smooth organisms. The mucoid antigen was not agglutinated by any of the serums.

In an attempt to determine whether previous infection with smooth *Brucella* would alter susceptibility to infection with variant *Brucella*, 11 guinea pigs were inoculated with 10^5 organisms of 2308S followed by approximately the same number of mucoid organisms of SMR2308M after 4 weeks. Animals were then sacrificed at weekly intervals and the colonial types present in the tissues identified (table 3).

TABLE 2.—*Persistence of* Br. abortus *strain SMR2308M in guinea pigs after simultaneous inoculation with strain 2308S.*

Animal number	Weeks after inoculation	Cultures isolated	Gross lesions	Serum titer with antigen	
				S	M
1	3	+S	−	p^{40}	−
2		++S, +M	−	p^{40}	−
3		+M	−	−	−
4	4	++S	+	20	−
5		+S	−	p^{20}	−
6		41S	−	−	−
7	5	+S, 12M	−	−	−
8		+++S, +M	+	160	−
9		++S	−	40	−
10	6	+++S	+	40	−
11		++S, 1M	−	p^{20}	−
12		+++S	−	p^{20}	−
13	7	++S	−	p^{20}	−
14		−	−	−	−
15		−	−	−	−
16	8	+S	−	5120	−
17		+++S	++	1280	−
18		+S	−	160	−
19	9	3S	−	320	−
20		+S	+	1280	−
21		9M	−	−	−

Inoculum: 10^5 organisms of SMR2308M and 10^3 organisms of 2308S.
S = smooth colonies; M = mucoid colonies; p = incomplete agglutination at that dilution.
If less than 50 colonies were recovered from an animal, the actual number of colonies is recorded, otherwise the relative numbers of organisms recovered are indicated by +, ++, etc.; negative results, −.

Although large numbers of smooth organisms could be recovered from the tissues of most animals, macroscopic pathological changes were observed in

animals were smooth. With the exception of one mucoid colony, all organisms recovered from animals examined at the 6th week were smooth. Cultures of tissues from animals 14 and 15 (7th week) did not yield *Brucella*. A few mucoid organisms were isolated from the prefemoral lymph nodes of guinea pig 21, sacrificed at the 9th week, but smooth organisms were not detected. The remainder of the animals in this series yielded smooth organisms only.

Two animals (2 and 4, sacrificed one and two weeks, respectively, after inoculation of the mucoid organisms) did not show evidence of infection with smooth organisms. Eleven mucoid colonies appeared on the plates inoculated with the spleen and right prefemoral

TABLE 3.—*Persistence of* Br. abortus *strain SMR2308M in guinea pigs inoculated 4 weeks previously with strain 2308S.*

Animal number	Weeks after inoculation of strain		Cultures isolated	Gross lesions	Serum titer with antigen	
	S	M			S	M
1	5	1	+S	–	–	–
2			11M	–	–	–
3	6	2	++S	–	p^{10}	–
4			+M	–	–	–
5	7	3	+++S	++	5120	–
6			+++S	+++	5120	–
7	8	4	31S	–	–	–
8			++S	+++	10240	–
9	9	5	+S	+	10240	–
10			+S	++	10240	–
11			++S	+	2560	–

Inoculums: 10^8 organisms of 2308S, followed in 4 weeks with 10^8 organisms of SMR2308M.
S = smooth colonies; M = mucoid colonies.
If less than 50 colonies were recovered from an animal, the number of colonies is recorded, otherwise the relative numbers of organisms recovered are indicated by +, ++, etc.; negative results, –.

lymph node of animal 2, and 60 mucoid colonies grew on the plate seeded with the iliac lymph nodes of animal 4. All other tissues of these two animals were negative upon culture. The other guinea pigs in this series were infected with smooth organisms only, and many displayed severe pathological alterations and high agglutinin titers for the smooth antigen.

Pathogenicity of five mucoid cultures

Five additional mucoid cultures that were streptomycin resistant were selected for a preliminary survey of their behavior in guinea pigs. Groups of 5 guinea pigs each were inoculated intraperitoneally with approximately 10^8 organisms of each mucoid culture. An animal from each group was sacrificed

TABLE 4.—*Pathogenicity for guinea pigs of 5 mucoid cultures of* Brucella.

Strain	Weeks after inoculation	Cultures isolated	Gross lesions
Br. suis SMR *suis* R^M	1	++R^M	–
	2	2 R^M ++++S	++
	3	1 R^M +++S	++
	4	+ R^M	–
	5	++++S	+++
Br. suis SMR *suis* M	1	++++M	+
	2	++++M	+
	3	++M	–
	4	++++M	epididymal abscess
	5	11M	–
Br. suis SMR Huddleson M	1	++M	–
	2	5M	–
	3	–	–
	4	–	–
	5	–	atrophic testes
Br. chortus SMR 6980 M	1	4M	–
	2	–	–
	3	–	–
	4	–	–
	5	++++M	epididymal abscess
Br. melitensis SMR 56741 M	1	+++M	–
	2	++++M	–
	3	++M	–
	4	+M	–
	5	+M	–

Inoculums: approximately 10^8 organisms.
M and R^M = mucoid colonies; S = smooth colonies.
If less than 50 colonies were recovered from an animal, the number of colonies is recorded, otherwise the relative numbers of organisms recovered is indicated by +, ++, etc.; negative results, –.

each week following the inoculation. The results are recorded in table 4.

Mucoid cultures appear to vary greatly in their ability to persist and produce changes in the tissues of guinea pigs. Some strains could be isolated only during the first few weeks after inoculation, while others persisted through the 5 weeks of observation. Reversion to smooth was observed with one strain (SMR *suis* R^M) in vitro and in vivo. Two strains (SMR *suis* M and SMR abortus 6980M) produced large epididymal abscesses. Strain *abortus* SMR6980M apparently was unable to persist in the reticulo-endothelial tissue or to disseminate beyond the local lesion in the epididymis, since mucoid organisms were not detected in other tissues in animal 5. As in all previous serological tests agglutinins were found only in the serums from animals infected with smooth *Brucella*, which arose by reversion.

Br. suis strain SMR *suis* R^M was chosen for more detailed study because of its ability to persist in the guinea pig and to revert to the smooth colonial form. Reversion had been observed during cultivation in vitro, but an attempt was made to introduce only mucoid cells in the inoculum for animals. The inoculum for 21 guinea pigs was prepared from a series of two single colony transfers and two agar slant transfers from which only mucoid colonies were obtained. Only mucoid colonies were seen in the final inoculum when it was plated directly and in plates for a viable cell count.

The progress of the infection as indicated by the relative numbers and

FIG. 1.—Pathogenicity for guinea pigs of *Br. suis* strain SMR *suis* R^M.

colonial types of organisms recovered from animal tissues is shown graphically in figure 1. Gross pathological findings have been reported in detail elsewhere (Jones, 1950).

Only mucoid organisms were recovered from the three animals sacrificed one week after inoculation. Hemorrhagic lesions in the body wall at the site of inoculation of animals 1 and 2, a testicular abscess in animal 1, and enlarged and hyperemic prefemoral lymph nodes in animal 3 suggested some toxicity inherent in the mucoid organisms.

Reversion to the smooth colony type was detected in two of three animals (4 and 6) examined two weeks after inoculation. All colonies recovered from the liver of animal 4 were smooth, those from the spleen were in the ratio of 1 smooth: 2 mucoid colonies, whereas all colonies from the lymph nodes were mucoid. Reversion to the smooth form also occurred in the epididymal abscess of animal 6, although over 90% of the organisms recovered from that lesion and all organisms recovered from the other tissues were of the mucoid type, with the exception of a few smooth colonies isolated from the tunica vaginalis.

Mucoid *Brucella* persisted without evidence of reversion to smooth in two animals (8 and 9) sacrificed three weeks after inoculation. Animal 7 examined at the third week was infected primarily with smooth organisms, although a few mucoid *Brucella* were recovered from the spleen and prefemoral lymph nodes.

Reversion had occurred in animals 10 and 11, sacrificed at 4 weeks, but the smooth form apparently had not become well established at that time. In contrast, animals 12 and 15 yielded large numbers of smooth organisms, and the mucoid type was entirely absent. Only smooth organisms* were recovered from the tissues of animal 14, with the exception of a large abscess found in the omentum, which yielded numerous mucoid and a few smooth colonies.

Large abscesses involving the seminal vesicles were found in animals 19 and 20, sacrificed at 7 weeks. Only mucoid organisms were recovered from these lesions, although smooth *Brucella* were present in the other tissues.

There was no evidence of reversion in animals 13, 16 and 21. Mucoid organisms could not be recovered from animals 13 and 21, and only a few persisted in the spleen of animal 16.

Reversion to smooth and almost complete elimination of the mucoid type was apparent in animals 17 and 18, as only a few mucoid organisms could be demonstrated in an epididymal abscess in animal 17.

Agglutinins for mucoid organisms were not detected in the serums of animals inoculated with SMR *suis* R^M, although the serums of individuals in which reversion had occurred agglutinated the smooth antigen.

DISCUSSION

The infectivity of a culture of *Brucella* should be considered in terms of the minimum number of organisms required for the establishment of an infection and the length of time the infection persists. Complete evaluation of a culture thus requires a titration utilizing

* Two types of smooth colonies were seen on the plate inoculated with the gastro-hepatic lymph node of animal 14. One colony type was translucent and blue-green in appearance, whereas the other was a more opaque yellow-blue colony. The difference was apparent only when the two colonial types were growing adjacent to each other on the same plate. Each of these two smooth types, upon inoculation into guinea pigs, was capable of infecting two out of three animals with an inoculum of 100 organisms, and three out of three with an inoculum of 1000 organisms. Similar colonial differences were observed in the cultures recovered from these guinea pigs.

sufficient numbers of animals which have been given varying numbers of organisms and examinations at successive time intervals. Although such a titration was attempted with only one culture, it was found that the numbers of organisms required to establish infection with the mucoid variant studied were greater than those necessary with the parent smooth type.

Mucoid cultures displayed variation in their pathogenic potentialities. Some strains could be recovered only during the first week after inoculation, while others persisted in the animal tissues for at least 9 weeks. A similar variation in the gross lesions produced was manifest.

The most extensive observations were made with *Br. abortus* strain SMR2308M and *Br. suis* strain SMR suis R^{M}. Comparison of the two cultures indicates that although both were capable of establishing an infection which persisted for 7 to 9 weeks, the *Br. suis* mucoid culture had the greater tendency to produce abscesses which did not show evidence of regression during the period of observation. A similar difference was seen between the two parent smooth types. Reversion from mucoid to smooth in vitro and in vivo was observed more frequently with SMR *suis* R^{M} than with SMR2308M. After infection with smooth organisms became established the *Br. abortus*, mucoid culture was eliminated from the animal tissues. On the other hand the *Br. suis* mucoid culture was able to persist within the local lesions it had produced, despite the presence of smooth organisms in the other tissues of the animal.

In simultaneous infections of SMR2308M and 2308S, the smooth form generally was found to persist longer. The numbers of both the mucoid and smooth strains given these guinea pigs had been shown in other experiments (Jones, 1950) to be incapable of establishing infection in 100% of guinea pigs. Thus, when inoculated together, either or both types could be eliminated by the host. In those individuals in which the smooth type became established, the mucoid type was present in small numbers or not at all. Mixtures of the two types could not be demonstrated after 5 weeks. However, mucoid organisms survived for as long as 9 weeks in an animal which did not show evidence of infection with the smooth strain.

Infection with strain 2308S established 4 weeks previous to the inoculation of mucoid organisms did not render animals more susceptible to infection with the mucoid form of the same strain. Mucoid organisms were recovered only from animals which did not present evidence of infection attributable to smooth organisms. Both types were never recovered from the same animal in this series. Apparently the mucoid organisms were eliminated more rapidly from animals which had been infected previously with the smooth culture than they were from animals which had been infected with the mucoid and smooth simultaneously or the mucoid alone. It is probable that part of the explanation for the more rapid elimination of mucoid organisms from animals previously infected with smooth organisms lies in the development of a specific immunity. The mucoid organisms thus persisted only in those animals in which the smooth inoculum had not been maintained long enough to stimulate refractivity. This does not explain the susceptibility to mucoid infection of animals displaying natural resistance to infection with the smooth form.

These findings are contrary to those reported by Braun (1950a), in which an intermediate type of *Br. suis* was able to establish itself in guinea pigs inoculated with a smooth culture containing less than 1% of an intermediate type.

Braun attributed the establishment of nonsmooth types in vivo to the disappearance of the smooth selective factor from the serum of infected animals.

From the limited data available, it is not possible to assess the relative importance for the establishment of variants of the disappearance of the selective serum factor and the establishment of a specific immunity, or if there is any relationship between the two. Further, the effect of prior infection with variant cultures on the susceptibility of animals to later infection with smooth cultures has not been studied intensively. Although Huddleson (1947) has reported that inoculation of guinea pigs with a variety of mucoid types engendered resistance to infection with smooth brucellae, it appears to be clear that prior infection with two of the mucoid types in this study (*Br. abortus* SMR2308M and *Br. suis* SMR *suis* R^M) failed to induce resistance to the smooth types which arose by reversion.

The differences in our results from those obtained by Braun and by Huddleson may be attributable to inherent differences among strains of organisms. Since the completion of this study, Braun (1950b) has reported similar results with mixed infections of mucoid and smooth *Br. suis*. Braun's intermediate culture had an ID_{50} very close to that of the parent smooth culture (Braun, 1951), while the ID_{50} of *Br. abortus* SMR2308M is about 1000 times greater than that of the parent smooth type.

Further observations on mixed infections were possible in guinea pigs in which the *Br. suis* SMR *suis* R^M inoculum reverted to the smooth form. In the absence of reversion, the numbers of mucoid organisms present in the tissues decreased with the passage of time, unless a local lesion had been established. When the smooth type appeared, it rapidly became the predominant form. After the first two weeks of infection, large numbers of mucoid organisms persisting in the presence of smooth organisms were always associated with a manifest focus of infection with the mucoid form. Mucoid organisms seemed unable to disseminate beyond the local lesion and its draining lymph nodes if smooth organisms were present in the spleen, liver, or other lymph nodes of the animal.

The *suis* R^M culture was capable of producing severe pathological changes of the same type as the parent smooth culture, particularly in the male sex organs, but its ability to persist at other sites seemed to be limited. It is interesting to speculate on possible differences in selective conditions within the tissues which appear to favor proliferation of variant types as compared to the tissues in which the smooth type may have been favored. The persistence of the mucoid type within well defined abscesses may be related to the accumulation of metabolites which influence selection as described by Goodlow et al. (1950).

The mucoid form of the *Br. suis* culture seemed to possess the toxicity of pathogenic strains, but it may lack other components of virulence necessary for invasiveness. Maaløe (1948) has reported an analogous case with an apathogenic variant of *Salmonella typhimurium*, which differed from the parent virulent form only in its greater susceptibility to the bactericidal activity of complement.

In his studies of mucoid variants in guinea pigs, Huddleson (1947) reported their elimination from the tissues by the 4th week of infection. The experiments described here indicate that mucoid cultures vary in their ability to persist in the tissues, to produce pathological alterations, and to revert to the smooth colony type. The rate of rever-

sion to smooth varies among strains and, furthermore, the frequency of the detection of such a reversion depends upon (1) the ability of the mucoid organisms to proliferate in the host in numbers sufficient for the occurrence of such a mutation, and (2) the presence of environmental conditions suitable for the establishment of the smooth mutant.

SUMMARY

The numbers of organisms required to establish infection in guinea pigs with the mucoid brucellae studied were greater than those necessary with the parent smooth types. Mucoid strains varied in their ability to persist in the tissues of guinea pigs. Some strains could be isolated only during the first weeks following inoculation, while others persisted for as long as 9 weeks.

Infection with several mucoid strains resulted in the production of large abscesses in the male sex organs. Moderate lymphadenitis and splenomegaly were observed early in the infection, but regression apparently took place with the passage of time.

The mucoid cultures studied did not stimulate the production of demonstrable agglutinins in the inoculated animals for either smooth or mucoid organisms. Reversion of one strain of *Brucella abortus* and one of *Brucella suis* from mucoid to smooth was observed in vitro and in vivo.

In mixed infections of mucoid and smooth organisms the smooth form was found to persist longer. The numbers of mucoid organisms present in the tissues decreased progressively unless a localized lesion was established, beyond which dissemination did not occur.

The mucoid form was eliminated more rapidly from animals which had an established infection with smooth organisms than from a similar series of animals not previously infected with the smooth form.

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THE EFFECT OF PITUITARY ADRENOCORTICOTROPHIC HORMONE ON EXPERIMENTAL TETANUS IN MICE

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A recent report[1] concerning the use of pituitary adrenocorticotrophic hormone in the treatment of rattlesnake bite, where the venom is hemolytic and has a histamine-like effect, and a black widow spider bite, where the venom is a nonhemolytic neurotoxin, suggested to us that ACTH might be of value as a therapeutic agent against other toxins. Therefore, experiments were carried out to study the effect of ACTH on mice inoculated with crystalline tetanal toxin.

METHODS

The crystalline tetanal toxin used in this study was supplied by Dr. Louis Pillemer.[2] Witte peptone (1%) in 0.9% saline solution was employed for diluting the toxin. The toxin was diluted and injected into the gluteal muscles of the mice at the base of the tail on the right side. White Swiss inbred mice weighing 25 to 30 g were employed. It was found that 0.5 ml of a 1:1,000,000 dilution of the tetanal toxin would produce death in all animals within 96 hours.

The ACTH (Acthar^{R}) was diluted with saline so that 1 ml of solution contained 1 mg of ACTH. Dosage employed was 2 mg daily given intraperitoneally in 4 equal doses every 6 hours.

Two types of experiments were done. In the first, the mice were injected with their first dose of ACTH one hour before the injection of tetanal toxin and the ACTH was administered every 6 hours thereafter. In the second, the ACTH was started 24 and 48 hours after the administration of tetanal toxin. Fresh dilutions of ACTH were prepared for each injection period.

TABLE 1.—*Effect of ACTH on experimental tetanus in mice.*

	No. of mice	Onset of symptoms	Death
Control tetanal toxin	20	18 to 24 hours	48 to 66 hours
Control ACTH	20		
Tetanal toxin + ACTH	20	18 to 24 hours	48 to 66 hours
Tetanal toxin + ACTH at 24 hours	10	18 to 24 hours	48 to 66 hours
Tetanal toxin + ACTH at 48 hours	10	18 to 24 hours	54 to 66 hours

RESULTS

The combined results are shown in table 1. In the experimental series all animals injected with tetanal toxin died sooner than did those used for determining the minimal lethal dose. This is presumably related to the frequent handling of the test animals. The signs of tetanus were similar to those described by Pillemer.[3] They consisted of contraction of

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We are indebted to Dr. Louis Pillemer and Dr. Kenneth Robbins for aid and advice concerning the organization of this experiment.

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the abdominal muscles on the right; contraction of the tail toward the right; spasticity of the right hind leg; progressive spasticity of all extremities; and, finally, convulsions and cyanosis followed by death.

The control animals that received ACTH alone showed no ill effects. There was no apparent difference in the time of onset of symptoms and the time of death between those animals receiving tetanal toxin alone and those animals receiving tetanal toxin and ACTH.

The apparent change in the lethal effect from 48 hours to 54 hours in those animals receiving ACTH 48 hours after an injection of tetanal toxing is not a real change, since this is of necessity a selected group consisting of animals which have already survived 48 hours.

SUMMARY

Pituitary adrenocorticotrophic hormone (ACTH) in dosage employed was found to have no therapeutic effect in mice inoculated with purified tetanal toxin.

THE RECOVERY OF L FORMS OF BRUCELLA AND THEIR RELATION TO BRUCELLA PHAGE

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L forms have now been described for several genera belonging to the families *Enterobacteriaceae*,[1–4] *Neisseriaceae*,[5] *Bacillaceae*,[6,7] and *Parvobacteriaceae*.[1,8–10] It has been suggested[11] that all true bacteria may produce such forms, providing the appropriate conditions are found. We wish to report the presence of these forms in yet another genus of the *Parvobacteriaceae*, namely, the *Brucella*.

EXPERIMENTAL

L forms recovered from human blood

During the course of work done on brucella blood cultures[12] and brucella phage,[13] it became increasingly evident that microorganisms comparable with the L forms noted above, in addition to the nonsmooth brucella variants already described, were being recovered from human blood specimens.

Using the technique for blood culture described by Pickett and Nelson,[12] one obtains a confluent mass of growth on the primary plate which continues to develop over a period of several days and which increases in opacity and quantity but which has been, until recently, impossible of subculture. That is, subcultures of this material would not develop in brucella broth or on secondary brucella agar plates,† but would, however, grow in thioglycollate medium (BBL). Occasionally, slight turbidity in broth or a questionable colony on the solid medium would develop, but these results were not reproducible. Because of these technical handicaps, it was impossible to state definitely that this secondary growth was that of *Brucella*. Primary cultures in broth of 0.1 ml blood sediment yielded a culture showing slowly developing turbidity, quite often granulated and almost always containing an ever increasing stringy, ropy mass which adhered to the bottom of the tube but which would swirl with agitation. This, too, could not be subcultured except into thioglycollate medium, which in turn would contain a fluffy mass of growth supported by the agar particles. The growth developed at a subsurface level and failed to occur in the colored top zone containing diffused atmospheric oxygen. Maximum turbid-

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† Albimi Brucella Broth enriched with 100 μg percent thiamine and 100 μg percent nicotinamide ("BTN" broth). This medium solidified with 2% of Difco agar ("BTN" agar).

ity occurred immediately adjacent to this zone, then decreased gradually towards the bottom of the tube. This is in agreement with Weinberger, Madoff, and Dienes.

Because of the large amount of blood, tissue extract, or agar present, none of these mediums which allowed growth was suitable for serological identification of this L form. It was therefore necessary to obtain a reasonably antigen free medium before such studies could be undertaken. We have now found that 5% peptic digest of blood[14] added to BTN broth serves as a medium suitable for such purposes. Human type "O" blood was used in order to eliminate possible Forssman reactions. A one loopful inoculum from a sediment broth can be carried through 5 passages in the peptic broth, in each case giving rise to a lightly turbid and occasionally granular growth.

Supernatants from such broth cultures give positive precipitin tests with anti-smooth *Brucella* serum and negative reactions with serums prepared against other brucella variants. It is apparent, then, that this L form is, at least antigenically, related to smooth *Brucella*.

Microscopically, this L form is comparable with L forms of other species in being highly pleomorphic, consisting of rods, cocci, chains, branching forms, various bizarre intermediate forms, ghost cells, and typical large bodies. These may be observed on slides stained with dilute carbol fuchsin (figures 1, 2 and 3). The L forms are seen in stained preparations obtained from primary sediment plates, primary sediment broths, and from peptic broths after several subcultures.

In addition to the peptic digest broth, we have prepared 5% peptic digest BTN plates (0.7% agar). These, though inadequate for luxurious growth, when inoculated with material from peptic digest broths, do give a sprinkling of typical L type colonies as described for other genera (figures 4 and 5). Secondary, large white opaque colonies quite often develop from the dark areas of the type of colony shown in figure 5. These coalesce until the small transparent L growth may be completely obscured. Microscopically, smears of this secondary colony reveal gram-negative cocci comparable to the SB variant.

L forms from stock smooth brucellae

Dienes, Weinberger, and Madoff suggest that L forms arise from typical bacterial forms when the latter are under conditions unsuitable for normal development. These would include exposure to antibiotics such as penicillin, host mechanisms such as antibodies and complement, inadequate mediums or tap water, temperatures other than optimum, and exposure to bacteriophage. They have recovered these forms under all of these conditions. It is the last of these which we feel most significant in the development of the L form from brucellae. Actually, many of the conditions found suitable for the development of L forms by other workers (autolysing cultures, antagonism of strains, presence of certain compounds) are conditions commonly seen as a result of or requisite to phage activity in bacterial cultures. Even penicillin has been shown to enhance phage activity.[15] Finally, it is recognized that phage often effects bacterial variation.[16–18] Accord-

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ingly, one might well expect to encounter phage in cultures exhibiting marked variation.

That phage is responsible for negative cultures in chronic brucellosis was suggested by Pickett and Nelson.[12] This was based on the fact that phage inhibitors were active in promoting growth from specimens and that the washing procedure used was effective in removing much of the phage shown to be present in the initial blood specimen. At this point, then, the conditions are propitious for the development of L forms, that is, both brucella phage and brucella organisms are present in the blood specimen.

Still other evidence supporting the "phage hypothesis" for the development of L forms is to be found in the fact that as the opaque mass of the L form develops on the primary plate, one also observes the development of varying numbers of plaques in this mass (figures 6 and 7). These plaques are inhibited if 100 μg percent acriflavine is included in the primary plate. When the plaques from such a plate are cored and enriched in a mixture of *Brucella suis*, *Brucella abortus*, and *Brucella melitensis*, filtrates are obtained which show high titer phage activity against one or more of the enrichment strains. Control mixtures of the three organisms do not yield such active filtrates. When dilutions of these filtrates are spotted onto plates inoculated with one of the enrichment strains by the spread technique, clearing occurs at the points at which the spots were placed (fig. 8). In addition, then, to the antigenic relationship between smooth brucellae and this L form, we may add the fact that phage, showing activity against the L form on primary culture, similarly shows low activity, capable of enhancement, against smooth brucellae. This is comparable to the work of Hale,[19] who has shown that small colony variants have the same serological and phage types as their parent strains.

Finally, if from these spot plates one prepares smears from the cleared zones of lysis which contain mainly debris and autolysing material, one observes all of the L forms described above, including the large bodies. Subcultures from the zones of lysis to thioglycollate broths yield L form growth typical for this medium. Subcultures from these thioglycollate broths to BTN plates yield a mixture of minute colonies and normal smooth colonies (fig. 9). These smooth colonies, when tested for sensitivity to the original phage, were found to be resistant. The small colonies when transferred to thioglycollate broths and to soft peptic digest BTN plates gave rise to L forms.

DISCUSSION

There has been much speculation concerning the role and significance of the L form.[1,4,20,27,28] It appears that this form may be essential to the survival of a bacterial species, not unlike the more familiar spores and cysts common to many classes of organisms. That this and allied forms exist in vivo has been shown.[20–24] Certainly, all the conditions common for the development of L

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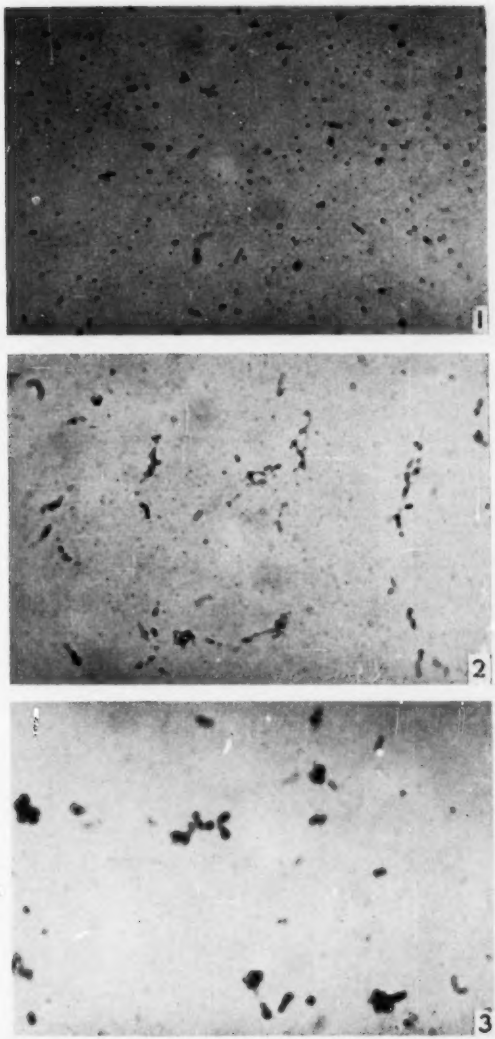
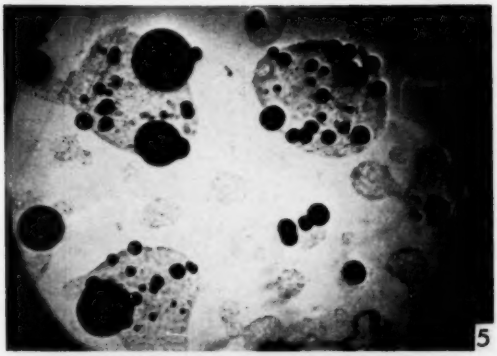


FIG. 1.—Pleomorphic forms and round bodies characteristic of growth in thioglycollate broth.
FIG. 2.—Pleuroneumonia-like forms characteristic of growth in 5% peptic digest BTN broth.
FIG. 3.—Pleomorphic organisms characteristic of L colonies on 5% peptic digest BTN–0.7% agar plates.



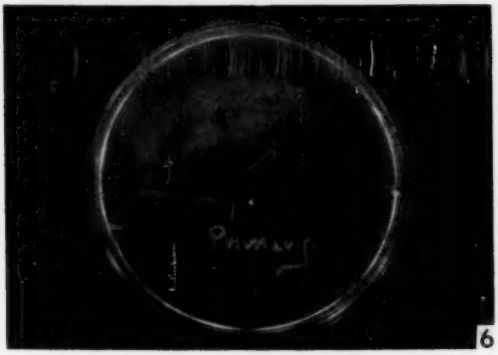
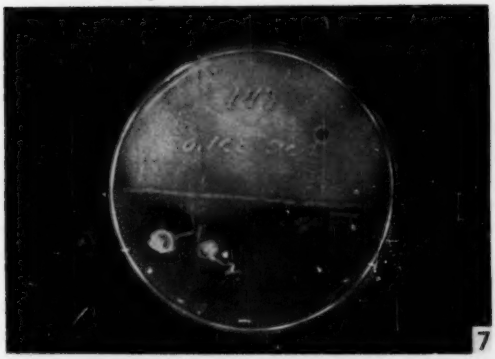
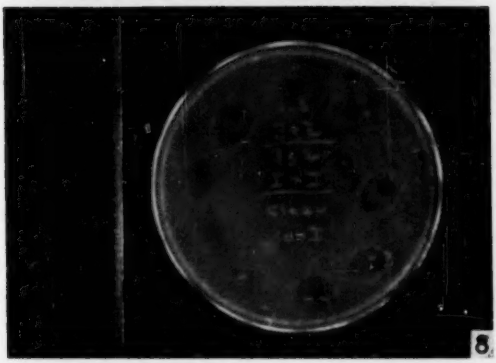


FIG. 4.—L colonies on 5% peptic digest BTN–0.7% agar plate inoculated from a thioglycollate broth (4 days, 35 C). ×20.

FIG. 5.—L colonies on 5% peptic digest BTN–0.7% agar plate inoculated from a 5% peptic digest broth (6 days, 35 C). × 20.

FIG. 6.—Primary sediment BTN plate of a clinically "normal" individual (4 days).





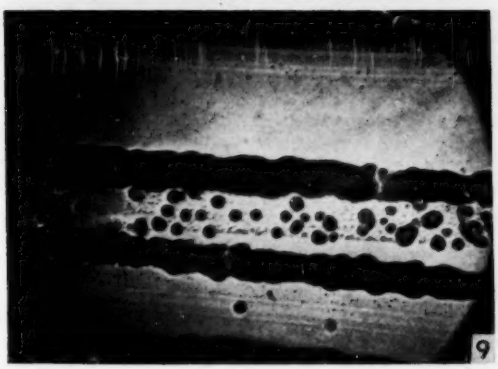


FIG. 7.—Primary sediment BTN plate of a patient with clinical symptoms of brucellosis (4 days).
FIG. 8.—Phage titration of anti-*suis* phage vs. *B. suis* strain 3S2. Titer approximately 10^{-6}.
FIG. 9.—Mixture of phage resistant smooth and minute colonies on a BTN agar plate (4 days, 35 C). ×20.

forms are present in vivo, especially when this environment includes therapeutic agents.[25,26] However, since all of the L forms reported for virulent bacterial strains have been shown to be nonpathogenic for the usual laboratory animals[4,7] and since conditions for their development in vitro are stringent, it is not surprising that the L forms should have been overlooked in the routine culture of clinical material.

That the presence of such a form would be important to an understanding of relapses and the carrier state is evidenced by its potential for giving rise to normal smooth organisms.

The observations presented here agree essentially with reports of L forms for other genera of bacteria. Main points of difference are their resistance to autolysis and their ability to grow in broth. The importance of brucella phage in the establishment of this form of *Brucella* is stressed because of their in vivo relationship. This suggests, then, a mechanism whereby many persons infected with *Brucella* show no clinical signs of brucellosis; that is, the suppression of smooth virulent *Brucella* by the propitious presence of brucella phage, leaves essentially avirulent variants, in particular the L form, as residue in the body. The variants, conceivably, on occasion give rise to virulent smooth *Brucella* which, if able to survive under the conditions obtaining, may initiate overt brucellosis.

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SUMMARY

L forms of the genus *Brucella* have been recovered from blood specimens and from cultures of smooth *Brucella* subjected to the action of brucella phage.

An antigenic as well as a phage relationship exists between the L form and smooth *Brucella*, the L form giving positive precipitin reactions with anti-smooth *Brucella* serum.

The possible role of this form and brucella phage in the epidemiology of brucellosis is discussed.

KINETIC ASPECTS OF HEMOLYSIS BY THE α-TOXIN OF *CLOSTRIDIUM WELCHII* AND ITS INHIBITION BY ANTITOXIN

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The activity of the bacterial hemolysins in vitro can be measured quantitatively by the spectrophotometric determination of liberated hemoglobin, and is specifically inhibited by homologous antibody. The antigen-antibody reaction can be made both quantitative and sensitive and should constitute a useful model for a variety of purposes. Exploratory studies with a number of bacterial hemolysins suggested that the α-toxin of *Clostridium welchii* might be one of the most satisfactory, in part because of its stability in dry form, and because it is one of the better known bacterial hemolysins.

This toxin has been differentiated from the other toxic components of filtrate of *Cl. welchii* cultures and has assumed some general interest because of its predominant role in the pathogenesis of infections by this microorganism, and because it is known to have, or be associated with, lecithinase activity. The literature to 1943 has been summarized by Oakley (1943).

Preliminary experiments indicated that the kinetics of α-toxin hemolysis and its inhibition by antitoxin are too poorly understood to allow the accurate titration of toxin and antitoxin. Macfarlane and Knight (1941), who established the enzymatic basis of the Nagler reaction and associated it with α-toxin, suggested that the lecithinase activity is responsible for the lysis of red cells, and this view has been echoed since by a number of other workers. While no direct evidence in support of this suggestion has been presented, it seemed reasonable to assume, as a working hypothesis, that α-toxin hemolysis is an enzyme-catalyzed reaction, and to approach the hemolysis and its inhibition on the basis of enzyme kinetics. The results of exploratory experiments are reported here.

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* With the technical assistance of Miss Dorothy E. Moore.

MATERIALS AND METHODS

Toxin.—Different lots of toxin, even from the same strain of *Cl. welchii*, differ to an appreciable extent in both hemolytic titer and ability to combine with antitoxin. To avoid this possible source of variability a single pool of toxin was used throughout the experiments reported here. One L of filtrate of liquid culture of *Cl. welchii*, type A, strain BG-6, lot number 481380, was made available through the kindness of the Lilly Research Laboratories. At the time of preparation, about 4 months prior to the initiation of the present study, it had a pigeon MLD of 24/ml. Within the range of concentration used here, it gave no hemolysis in isotonic phosphate buffer, which was taken to indicate that the hemolytic activity was that of the α-toxin alone. The filtrate was dialyzed against running tap water for 18 hours, lactose added to 1% and the solution lyophilized. As noted by van Heyningen (1941) and confirmed in preliminary experiments here, hemolytic titer was not affected by this treatment. The lactose served only as a dry diluent of the final material. The yield of hygroscopic light brown powder was 12.69 mg/ml of the solution and contained approximately 80% lactose. It was stored in small vials in a vacuum desiccator and under these conditions was stable over a period of at least 10 months. The 50% hemolytic dose (HD_{50}) was 2 to 4 μg in the present titrations; in the present report no correction is made

for the lactose diluent. This activity is equivalent to about 4000 HD_{50}/ml of the original filtrate and about 4 times that of van Heyningen's (1941) filtrates, which contained approximately 1000 HU(50% hemolytic units)/ml. Solutions in isotonic saline had a pH of about 6.0, and the material was readily soluble in isotonic saline containing M/100 buffer from pH 5.0 to 8.0.

Others have found that α-toxin deteriorates rapidly in dilute solution, and we have observed a 20 to 30% reduction in hemolytic activity in 24 to 72 hours in isotonic saline solutions of the above material in concentrations of 1 mg/ml. We found, however, that 0.1% bovine albumin (Armour, Fraction V) not only stabilized the activity of this toxin dilution for at least 2 weeks at refrigerator temperature, but also resulted in about a 5 to 10% increase in the titratable activity of freshly prepared dilutions, suggesting that appreciable loss of activity occurred in dilute solutions very soon at 37 C. The significance of calcium to hemolysis by the α-toxin has been shown by Oakley and Warrack (1941), van Heyningen (1941) and others. Taking Oakley and Warrack's (1941) dilution factor into consideration, the lower limit of maximal hemolysis was at the equivalent of 0.008 to 0.016% calcium. In the experiments reported here 0.0025 *M* calcium acetate in the final mixture is the equivalent of 0.010% calcium and the same final molarity used by van Heyningen (1941). For use, then, stock toxin was prepared at weekly intervals as a solution containing 1 mg/ml in isotonic saline containing 0.1% bovine albumin and 0.0025 *M* calcium acetate. This procedure gave highly reproducible results over a period of many months.

Antitoxin.—The antiserum was a bivalent gas gangrene antitoxin, lot number 474,343, obtained from the Lilly Research Laboratories. It was a solution of salted out globulin, contained 700 units of *Cl. welchii* antitoxin and 800 units of *Cl. septicum* antitoxin per ml, and was preserved with 0.25% phenol and 1:20,000 merthiolate. It was strictly homologous in that the toxin described above was the immunizing antigen, first as toxoid and later as unmodified toxin. All antitoxin dilutions were made in calcium acetate-saline, and were prepared immediately prior to use.

Red cell suspensions.—Rabbit red cells were used throughout the experiments reported here. Fragility of the cells in the presence of α-toxin appeared to vary from one animal to another and from one time to another in the same animal, but the variation was of a relatively low order and is not considered here. Nevertheless, red cells from the same animal were used in groups of experiments in which small differences in hemolysin titer were compared. The animals were bled from the marginal vein of the ear into an equal volume of isotonic citrate solution and the cells washed three times in calcium acetate-saline and suspended in this solution for use. Cell suspensions were freshly prepared for all titrations. They were standardized photometrically at 540 mμ on the basis of contained hemoglobin liberated by saponin hemolysis. In preliminary standardization 1 ml of red cell suspension was diluted with 1 ml of isotonic saline, and 1 ml of 0.33% saponin was added to give a final concentration of 0.1% saponin as recommended by Ponder (1948). Lysis was essentially instantaneous.

Measurement of hemolysis.—In the titrations the total hemoglobin, representing both 100% hemolysis and red cell concentration, and that liberated by partial hemolysis produced by α-toxin, were determined as ferri hemic acid after separation from stroma and unlysed cells by centrifugation and expressed as the equivalent of hemin in μg. The conversion of the liberated hemoglobin to a stable heme compound was necessitated by degradation of oxyhemoglobin to hematins in the presence of the toxin. This occurred fairly rapidly and within an hour of decanting the absorption spectrum had shifted sufficiently to make measurement at a single wave band impractical. Since the toxin preparation was purified only to the extent of removal of dialyzable substance, it contained any stable soluble enzymes secreted by the bacteria during growth, and it seems probable that such contamination rather than the α-toxin itself was responsible for the observed changes in hemoglobin.

The hemoglobin in solution was converted to ferri hemic acid by the addition of hydrochloric acid in acetone solution as described by Morrison and Anderson (1942). With the amounts of hemoglobin used, 1 ml of a 2.5% solution was sufficient to precipitate the globin and maintain the heme in solution. The precipitated globin was so small in amount that the optical density was not affected to a detectable degree, and antitoxin and bovine albumin were sufficiently dilute so that the serum protein precipitated was also negligible; consequently, centrifugation prior to reading was not necessary. The cuvettes were tightly stoppered and allowed to stand at room temperature for one hour to insure complete conversion, and the color was stable for at least 24 hours.

The relation of erythrocyte concentration as hemin equivalent to numbers of cells was given

by a series of cell counts, and it was found that the hemoglobin liberated by saponin lysis of approximately 180×10^6 cells was the equivalent of 100 μg of hemin. In practice a suspension of about 3% packed cells, when diluted 1 part in 3, or 1 part in 4, gave a concentration of oxyhemoglobin and ferri hemic acid, respectively, near the upper spectrophotometric limit. Lower concentrations were prepared by dilution. It was found too time consuming to replicate red cell concentrations within less than 5%.

Titration.—Hemolysin titration was carried out in a total volume of 3 ml, made up of 1 ml of buffered saline or antitoxin dilutions in buffered saline, 1 ml of toxin dilution, and 1 ml of red cell suspension. Concentrations of all these are given below as concentration of the added solution or suspension, i.e., the total amount present, and not as a concentration per ml in the final mixture. Except where otherwise specified, the saline was buffered with *M*/100 acetate at pH 6.0. The titrations were carried out in the following steps:

1. Buffered saline, i.e., isotonic saline containing 0.0025 *M* calcium acetate and buffer to *M*/100 concentration, plain or containing antitoxin dilutions, was distributed in 1 ml amounts in 18×100 mm heavy pyrex tubes. Five nonphosphate buffers were used: veronal, acetate, glycylglycine, alanine, and tris (hydroxymethyl) amino methane (Gomori, 1946); the last is referred to hereafter as tris. The tubes and contents were then chilled to 2 C in a constant temperature bath.

2. Serial dilutions of toxin in bovine albumin-calcium acetate-saline, freshly prepared from the stock solution described above, were then added in 1 ml amounts to each of the tubes containing chilled buffer. In experiments in which the toxin-antitoxin mixtures were incubated, the contents were thoroughly mixed, the tubes transferred to a 37 C constant temperature bath, incubated for the appropriate time, and then chilled again prior to the addition of red cells. Control experiments showed that there was no detectable deterioration of toxin alone by this procedure with 37 C incubation as long as 90 minutes.

3. Chilled red cell suspension of the required density was then added to the above mixture in 1 ml amounts. It was found advantageous to pipette the cell suspension from a container immersed in ice water and to keep the suspension gently agitated with a magnetic stirring apparatus. The use of chilled reagents to this point was essential for reasonably accurate determination of the velocity of the 37 C phase of the reaction, and control experiments showed that any reaction occurring at 2 C prior to 37 C incubation, over the period of time consumed in so setting up the titration, was negligible.

4. After mixing, the tubes were transferred to 37 C and incubated for the specified time, with mixing at 10 minute intervals during this incubation. They were then returned to the 2 C bath and held for 30 minutes.

5. The tubes were then centrifuged at 3500 r.p.m. to spin down and pack the unlysed cells and stroma so that the supernate could be decanted and drained into cuvettes. These were 12×100 mm pyrex tubes matched to within 1%. One ml of 2.5% hydrolhloric acid in acetone was then added, the cuvettes tightly stoppered and inverted for thorough mixing, and the ferri hemic acid determined after one hour at room temperature. In some titrations it was measured at 540 mμ in a Beckman DU spectrophotometer, but the majority of determinations were made in Klett-Summerson photoelectric colorimeter, using a no. 54 filter.*

EXPERIMENTS

The study reported here was concerned with the relationships between toxin concentration, antitoxin concentration, and red cell concentration, and the effect on these of 37 C incubation time; the last allowed measurement of the velocity of the warm phase of the reaction. The warm phase was held constant with regard to temperature, i.e., temperature coefficients were not determined, and cold incubation (2 C) was held constant as to both temperature and time (30 minutes). Hemolysis was measured both as percent hemolysis and as absolute hemolysis, the latter as the hemin equivalent in μg of hemoglobin liberated regardless of red cell concentration. All parameters were determined by least squares.

The relation between toxin concentration and percent hemolysis on arithmetic plot was found to be described by an S-shaped curve that was markedly

* The cuvette described does not fit the test tube adapter of the Klett. Adapters to take cuvettes over a considerable size range are readily constructed in the usual shop, and such an adapter has been used here.

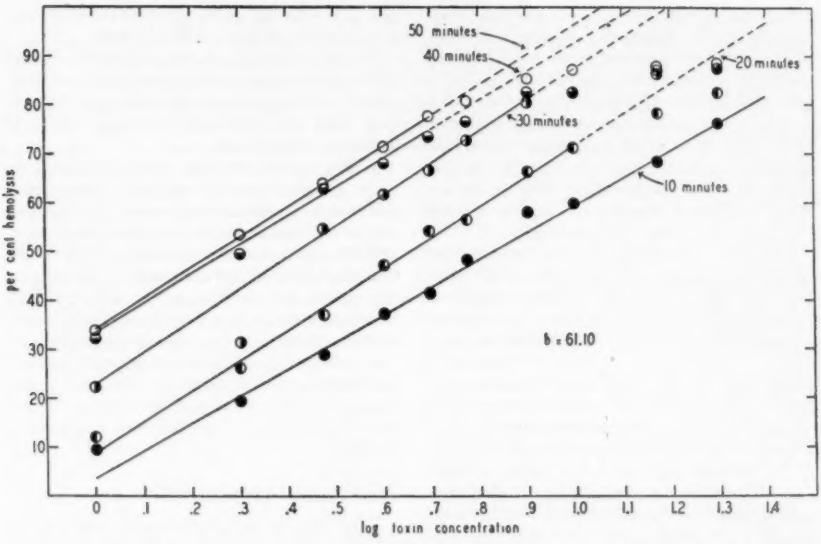

FIG. 1.—The relation between percent hemolysis and log toxin concentration; acetate buffer, pH 6.0, red cell hemin equivalent 164.1. The warm phase incubation time is given for each titration, and the indicated slope, b, is the average of the data illustrated.

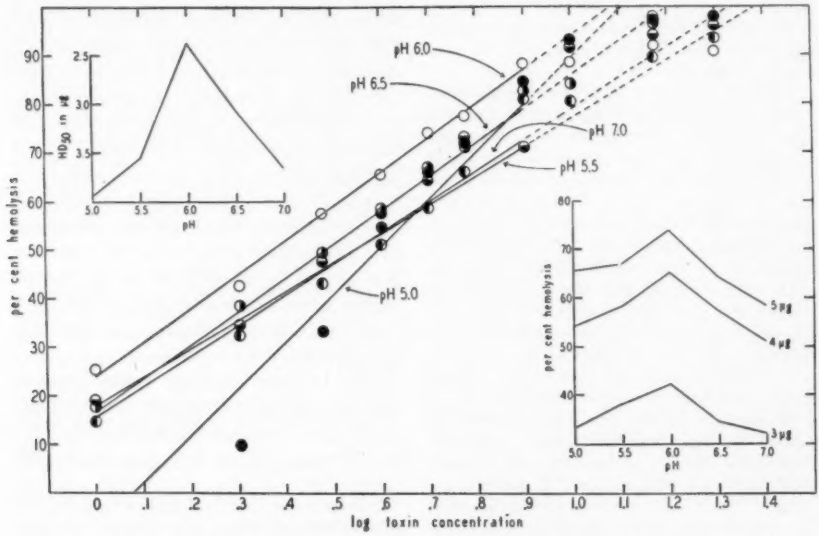

FIG. 2.—The effect of pH, over the range 5.0 to 7.0 in steps of 0.5, on the percent hemolysis-log toxin relation; acetate buffer, red cell hemin equivalent 160.0. The pH of each titration is indicated, typical titration data are shown in the lower right, and the effect of pH on the HD_{50} in the upper left.

asymmetrical, with the point of inflection near the lower asymptote. When percent hemolysis was plotted against the log of the toxin concentration in μg, the relationship became essentially linear between the limits of 20 to 70% hemolysis. This is illustrated in figure 1, which also shows that the slope of the linear portion of the curve was independent of 37 C incubation time over the range of 10 to 50 minutes. In this group of titrations the average slope was 61.10. In single titrations this value was reproducible to within about 5% and the observed extremes have been 57 and 63. If the data of figure 1 are used to calculate the HD_{50} dose of toxin and the dose plotted against time of incubation, the curve is still rising relatively rapidly at 30 minutes, but at 40 minutes it is becoming flat; the incubation period of choice so far as minimizing time error is concerned would appear to be 40 minutes. Below 15 to 20% and above 70% hemolysis the percent hemolysis-log toxin relation became nonlinear and seemingly asymptotic to about 10% and 85%, respectively. The lower asymptote is apparent here only in the 10 minute data but was a constant occurrence under appropriate conditions. These data show that differences in toxin titer associated with incubation time were determined by displacement of the entire curve rather than differences in slope of the linear portion.

The optimum pH for α-toxin hemolysis does not seem to have been defined with precision, and there are some discrepancies in the published reports. Most workers have used borate buffer at pH 7, but Oakley and Warrack (1941) observed that the MHD (minimum hemolytic dose, that giving the faintest detectable hemolysis) was greater at pH 8 than at pH 6. Here the effect of pH over the range 5.0 to 8.0 in the buffers indicated above was examined with respect to the percent hemolysis-log toxin relation. The results with all the buffers were essentially the same; titration in $M/100$ acetate over the range 5.0 to 7.0 is shown in figure 2. The slope of the linear portion was not appreciably affected except at pH 5.0, where it was significantly increased. This was possibly due to an increased red cell fragility suggested by traces of hemolysis in the controls and was probably not an effect of acetate ion, since it occurred in the other buffers. Differences in the HD_{50} shown in the upper left of figure 2 are attributable to displacement. Both the HD_{50} and the representative titration data shown in the lower right indicate an optimum at pH 6.0. Other titrations over a narrower range in steps of 0.2 showed that the optimum was relatively sharply defined in the region of pH 5.9 to 6.1.

The effect of red cell concentration on the hemolytic activity of the α-toxin was assayed as percent hemolysis and absolute hemolysis in relation to log toxin concentration. Representative data are shown in figure 3 for red cell concentrations varying from 74.8 to 204.8 μg hemin equivalent. The relation between percent hemolysis and log toxin concentration was remarkably constant as shown on the right, but the displacement that is evident has the practical consequence of a depression of the 50% endpoint with increasing red cell concentration, to give an inverse relation between the two. This is illustrated in figure 4 and it is clear from these data that the depression is independent of toxin concentration over a 4 fold range. Some workers have suggested the use of a heavy suspension of red cells in the hemolysis titration, such as equal volumes of toxin dilution and 6% red cell suspension; this is approximately a hemin equivalent of 720 in the

titration procedure described here and would result in a marked depression in a hemolytic endpoint.

On the basis of absolute hemolysis, shown on the left in figure 3, the activity was sharply increased by an increase in red cell concentration, rising 2.9 fold with a 2.2 fold increase in red cells. The effect of toxin concentration was also in sharp contrast to that measured by percent hemolysis and is illustrated in figure 5. Here the direct relation of absolute hemolysis-log toxin to red cell concentration was also directly related to toxin concentration, a 4 fold increase in toxin concentration resulting in a 3.2 fold increase in the effect of red cell concentration. The kind of buffer in which the titrations were carried out was found to be of considerable significance. The direct linear relation between absolute hemolysis and red cell concentration shown in figure 5 held true only in acetate buffer. In the other 4 buffers the relation, though not significantly different in slope from that observed in acetate, was linear only to a hemin equivalent of approximately 150, and fell off at higher red cell concentrations.

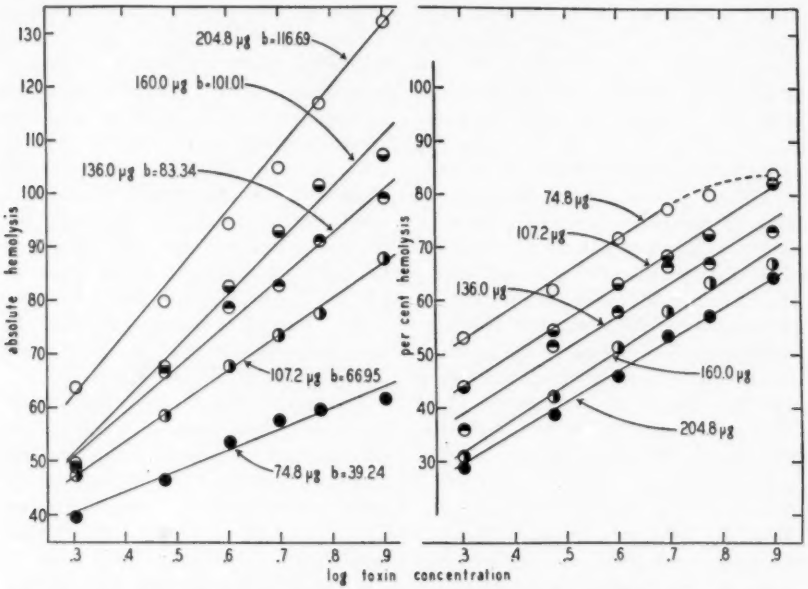

FIG. 3.—The effect of varied red cell concentration on the relation between absolute (left) and percent (right) hemolysis and log toxin concentration; acetate buffer, pH 6.0. The red cell concentrations are given as hemin equivalents, and indicated for each titration series. On the left the slopes of the fitted lines are given as values of b.

The velocity of the warm phase of the reaction was measured as absolute hemolysis per unit time, with the incubation periods in the over-all range of 10 to 50 minutes, depending upon toxin concentration and other factors. The time-hemolysis relation was linear on arithmetic plot over the 20 to 70%

range of hemolysis, with an early latent or induction period, and becoming asymptotic beyond 70% hemolysis. The data shown in figure 6 are typical and illustrate the velocity of hemolysis by toxin varying in concentration from 2 to 8 μg, the widest range compatible with accurate assay of liberated hemoglobin, in the presence of 160.0 μg hemin equivalent red cell concentration. The velocity is measured as the slope, b, of the fitted lines. The small but consistent increase in velocity with increase in toxin concentration is apparent, and the relationship between the two was linear on arithmetic plot with a slope (b/μg toxin) of 0.230. This contributes in only a minor degree to titratable activity, and what has been referred to earlier as displacement accounts almost entirely for differences in the hemolytic activity of different toxin concentrations. Here it is clear that the displacement is a consequence of the inverse relation between toxin concentration and the length of the induction period.

The velocity of the warm phase in acetate buffer was a little more than twice that in any of the other buffers used. This is illustrated in figure 7. In this respect, acetate appeared to be the buffer of choice for velocity studies and particularly so as regards the effect of substrate concentration on velocity, since, as indicated above, only in acetate was the relation of substrate concentration to hemolysis linear over the entire range.

The effect of red cell concentration on

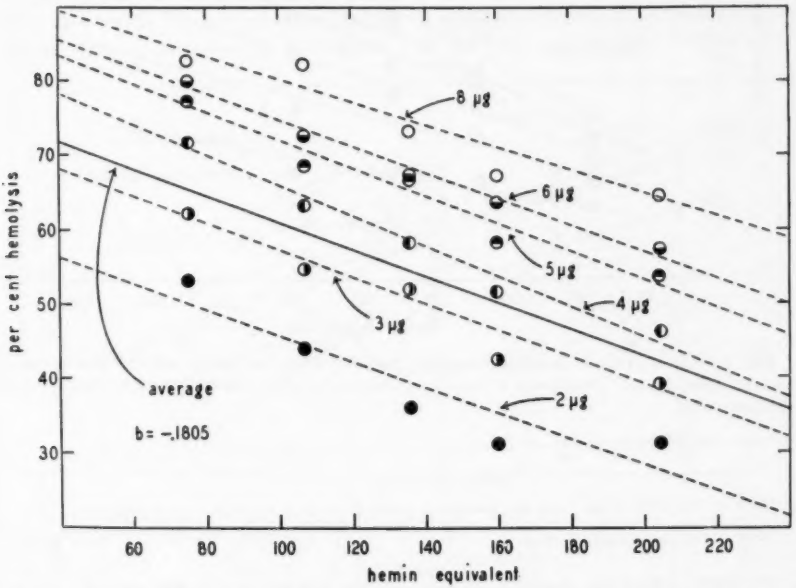

FIG. 4.—The depression of percent hemolysis by increasing red cell concentration; acetate buffer, pH 6.0. The broken lines are fitted to the points shown, and the solid line is an average, using the average value of b but an arbitrary intercept. The amounts of toxin are indicated in μg for each titration group.

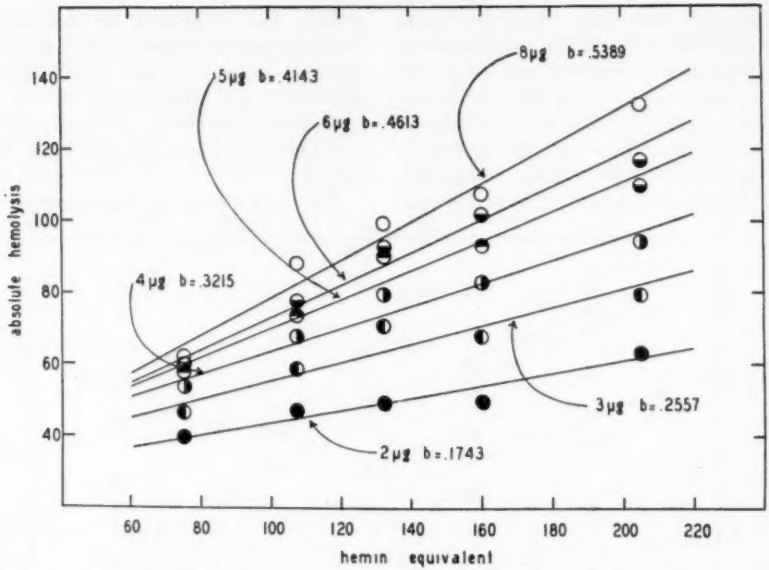

FIG. 5.—The increase in absolute hemolysis resulting from increase in red cell concentration; acetate buffer, pH 6.0. The amount of toxin in μg and the slope of the fitted line are indicated for each titration series.

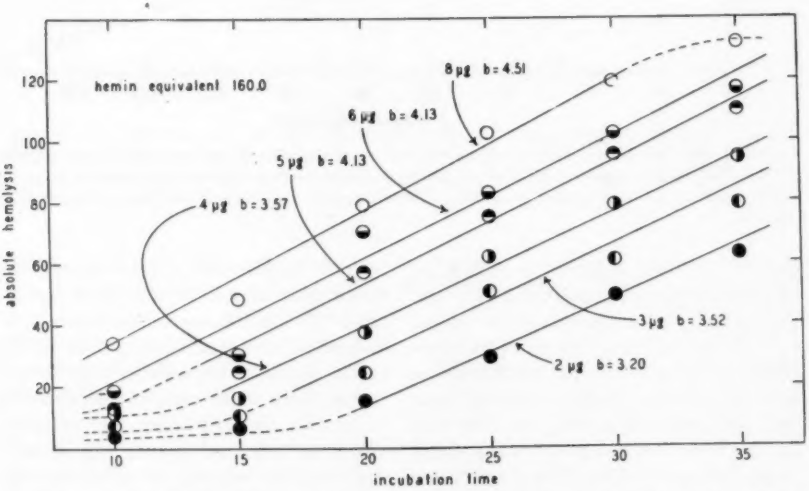

FIG. 6.—Velocity of the warm phase reaction measured as μg hemin equivalent of hemoglobin liberated per minute. In each titration series the amount of toxin is shown in μg, and the velocity as b, the slope of the fitted line.

velocity was marked and is illustrated on the left in figure 8. The accelerating effect was direct and essentially a 1:1 relation. In the data shown here, for example, a 2.74 fold increase in red cell concentration is associated with a 2.76 fold increase in velocity.

The inhibition of α-toxin hemolysis by antitoxin was of interest, particularly as regards the nature of the inhibition. It was approached through a study of the effect of antitoxin in underneutralized mixtures on the relationships described above. Neutralization is commonly affected by incubation of toxin-antitoxin mixtures prior to assay of the remaining activity. Here the toxin-antitoxin incubation time at 37 C was varied up to 90 minutes prior to the addition of red cell suspension. The data shown in figure 9 are typical of the results of assay on the basis of the percent hemolysis-log toxin relation; in this group a red cell suspension having a hemin equivalent of 180.8 μg and a constant amount of antitoxin, 1:200,000 dilution expressed as the reciprocal of the dilution or 5γ, were used. The resulting curves are similar to those obtained with toxin alone, consisting of a latent portion, a linear relation between 20 and 70% hemolysis, and thereafter asymptotic. The slope was not markedly affected other than some increase after 60 minutes incubation. Again the decreasing activity with increasing incubation time was primarily a function of displacement resulting from variation in the lower asymptotic portion of the curve, though increased slope of the linear portion at 60 minutes and after contributes in part to counteract the effect of displacement. That the apparent increased hemolytic efficacy of unneutralized toxin is other than an artifact is shown by other observations recorded below.

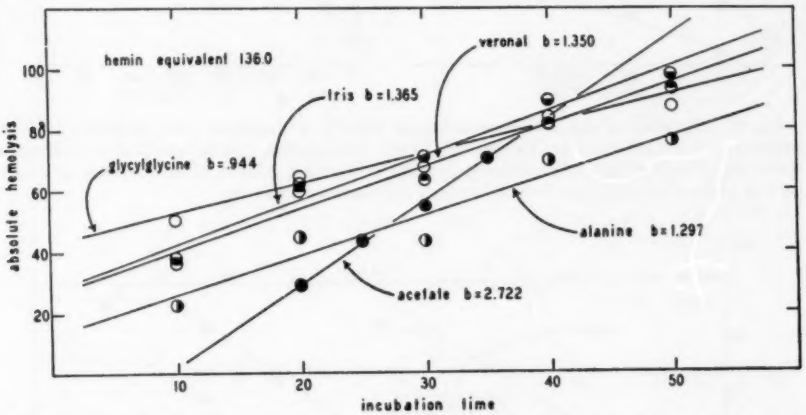

FIG. 7.—Velocity of hemolysis by 6 μg of toxin in various buffers. The buffers and velocities are indicated for each titration series.

Plot of the HD_{50} dose against toxin-antitoxin incubation time showed a rapid rise in neutralization which leveled off at around 60 minutes to become asymptotic, and the form of the curve resembled that of an adsorption isotherm. If this may be taken to represent the rate of reaction between toxin and antitoxin, it appears that this occurred

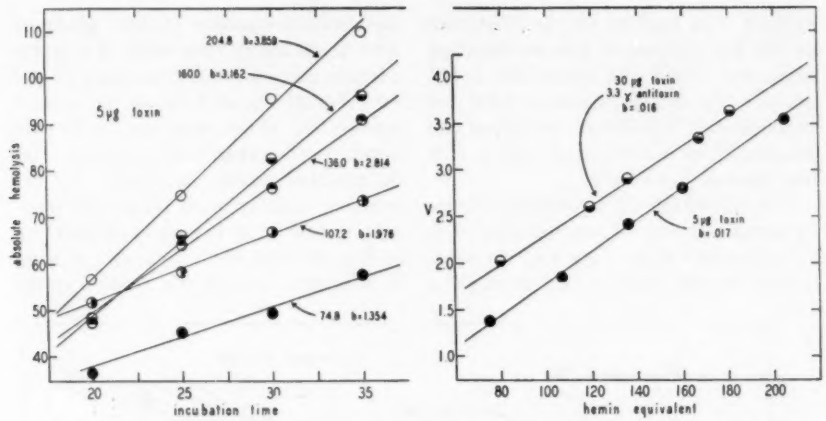

FIG. 8.—The effect of red cell concentration on velocity of hemolysis. Left, hemolysis by 5 μg toxin; the red cell concentration as hemin equivalent, and velocity, b, is indicated for each titration series. Right, illustrating the linear relation of velocity of hemolysis to red cell concentration, produced by 5 μg toxin alone and by an underneutralized toxin-antitoxin mixture.

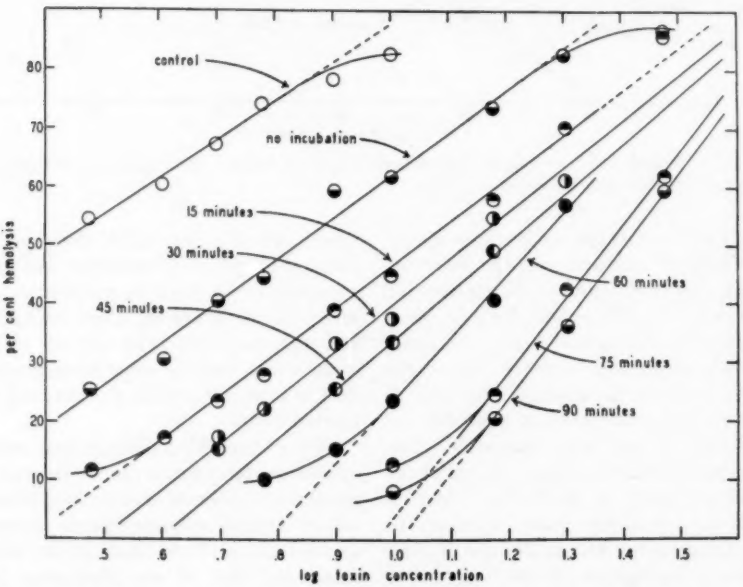

FIG. 9.—The effect of incubation time of toxin-antitoxin mixtures on the percent hemolysis-log toxin concentration relation; red cell concentration 180.8 hemin equivalent, antitoxin 5γ (1:200,000). The toxin-antitoxin incubation time is indicated for each titration series.

more slowly than might have been anticipated. Subsequent study of the behavior of underneutralized mixtures was carried out with no incubation and with 60 minute incubation of toxin-antitoxin; only the 60 minute incubated mixtures are considered in this report.

The effect of varied red cell concentration on the velocity of hemolysis produced by underneutralized toxin-antitoxin mixtures was similar to that on the velocity of hemolysis in the absence of antitoxin, but the velocities were consistently greater in the presence of antitoxin. Plot of absolute hemolysis against incubation time was distinguished from that shown on the left of figure 8 only in the slope of the lines. This is illustrated on the right in figure 8 in the plot of velocity, v (b), against hemin equivalent where an underneutralized mixture containing 30 μg of toxin and 3.3γ of antitoxin is compared with 5 μg of toxin alone.

The degree of neutralization was found to affect materially the absolute hemolysis-log toxin and percent hemolysis-log toxin relations. A typical series of titrations is shown in figure 10. Here the control indicates slope only and has been given an arbitrary intercept to eliminate displacement toward the left. It is evident that the slope is increased in the presence of antitoxin, and that the increase is directly related to anti-

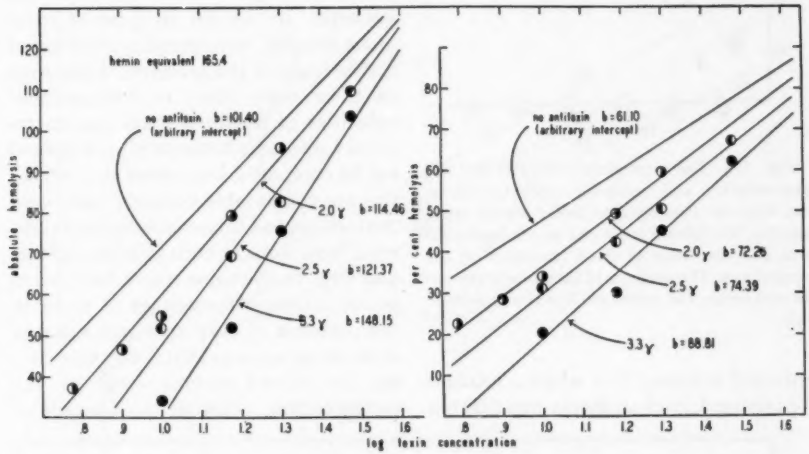

FIG. 10.—The effect of antitoxin concentration on the relation of log toxin concentration to absolute and percent hemolysis. Amounts of antitoxin and slopes of fitted lines are shown for each titration series. The same data are shown for absolute hemolysis on the left and percent hemolysis on the right.

toxin concentration. This effect of antitoxin was found to be related also to red cell concentration and is shown in figure 11, in which slope (b) is plotted against the hemin equivalent of red cell concentration.

The apparent velocity of hemolysis by underneutralized mixtures of toxin and antitoxin was found to vary widely according to the degree of neutralization, and, when the mixtures were only slightly underneutralized, the 37 C phase of the reaction had to be extended to allow appreciable hemolysis. Complete neutralization, then, could be regarded as an indefinitely great extention of the induction period. This is il-

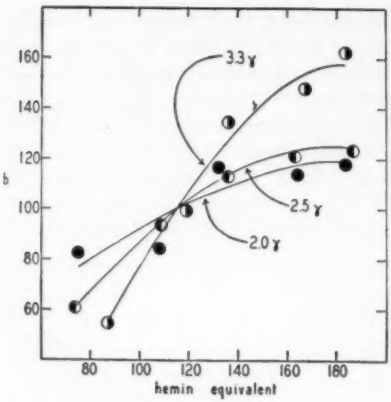

FIG. 11.—The interrelated effect of red cell concentration and antitoxin concentration on the slope of the absolute hemolysis-log toxin relation. The former is plotted on the horizontal axis, and the latter as the b constants on the vertical axis. The amounts of antitoxin are shown for each series. The curves are fitted by inspection only.

lustrated in figure 12, in which a mixture containing 3.3γ of antitoxin was titrated against toxin concentrations varying from 3 to 40 μg; only 6 of the toxin concentrations are shown. Here the 37 C phase was extended for the lower toxin concentrations. It is probable that the observed velocities for these low toxin concentrations are spurious in that they represent the induction period shown in figure 6, while in higher concentrations the velocities are similar to those of toxin alone roughly corresponding to those of active toxin in the mixtures. Data such as these were taken to indicate that velocities of identical toxin concentrations with and without antitoxin should not be compared, but rather that velocities are comparable between toxin concentrations and toxin-antitoxin mixtures when hemolysis in both falls between 20 and 70% in the same warm incubation period. Increased amounts of toxin in the presence of 3.3γ antitoxin resulted in an increase in velocity, but this cannot be related unequivocally to the corresponding effect in the absence of

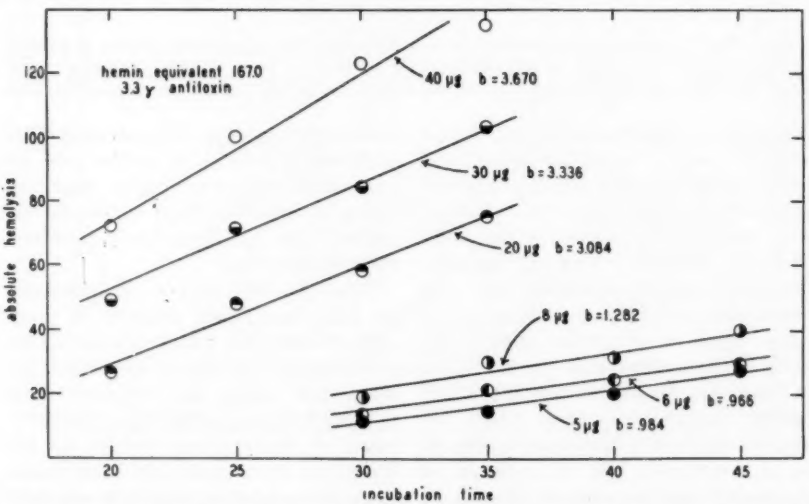

FIG. 12.—The velocity of absolute hemolysis produced by underneutralized mixtures of 3.3 γ of antitoxin and the indicated toxin concentrations in μg. Velocity for each titration series is given as b.

antitoxin. The slope of the fitted line (b/μg toxin) was reduced from 0.230 in the absence of antitoxin to 0.034 for the underneutralized mixtures illustrated here. This reduction appears to be a scale effect in that a 2 fold increase in toxin concentration in the underneutralized mixture resulted in a 0.57 increase in velocity, while with toxin alone the corresponding velocity increase was 0.45. If the fold increase of toxin in the toxin-antitoxin mixtures were considered as a fold increase in toxin in excess over that indeterminate amount completely neutralized, the increase in velocity would be much greater. These observations may, then, be taken to suggest that the velocity of hemolysis is increased by the presence of antitoxin in underneutralized mixtures.

In general the neutralization of soluble toxins by homologous antitoxin occurs in multiple proportions. Disregarding the variable effect of antitoxin on the several aspects of the α-toxin hemolysis described above, it was of some interest to find that neutralization of hemolytic activity was linear. This is illustrated in figure 13, in which the HD_{50} in μg is plotted against antitoxin concentration for 3 red cell concentrations. Inhibition was found to be a function of the antitoxin:toxin ratio, and the relation between this ratio and percent inhibition of percent hemolysis was also approximately linear. This is shown in figure 14, in which the ratios were taken from a wide range of concentrations, 3 to 40 μg of toxin and 1.7 to 5.0γ of antitoxin; i.e., dilution was not a factor. Data are given only for inhibition greater than 50% to show

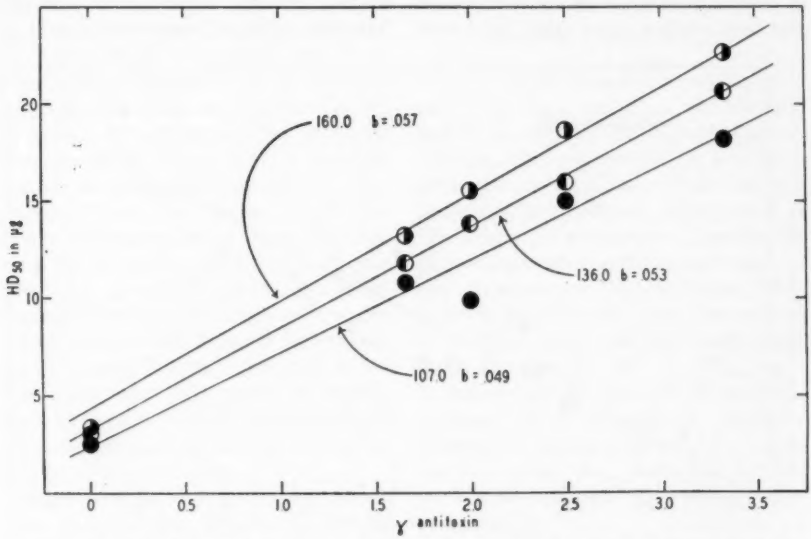

FIG. 13.—Antitoxin inhibition of hemolysis measured as increase in HD_{50} with increase in antitoxin concentration. The effect of red cell concentration is shown by the three groups of titration series. The difference in slope is probably a reflection of the effect of antitoxin on the percent hemolysis-log toxin relation (fig. 10), since it is not affected by variation in red cell concentration alone (fig. 3). It is clear that error in antitoxin titration is minimized by using the lowest practical concentrations of antitoxins.

that it does not become asymptotic to the antitoxin:toxin ratio even up to 95%.

DISCUSSION

The direct effects of increasing toxin or red cell concentration on the velocity of α-toxin hemolysis are consistent with the assumption that the hemolytic activity has an enzymatic basis but are in no sense unequivocal proof of it. The α-toxin of *Cl. welchii* is not unique in this respect. Herbert and Todd (1941), using a method of absolute hemolysis assay described by Herbert (1941) and closely similar to that reported here, found that the activity of streptolysin O, measured as the amount of hemolysin giving 50% hemolysis, was directly related to red cell concentration. In studies on hemolysis by a number of bacterial and other hemolysins at constant red cell concentration, Bernheimer (1946) showed that the velocity of hemolysis was directly related to lysin concentration. The relation was linear for streptolysin S′, *Clostridium septicum* hemolysin, pneumolysin, θ-toxin of *Cl. welchii*, and tetanolysin; anomalous for streptolysin O; and exponential for tyrocidine and a number of nonbacterial hemolysins such as saponin and taurocholate. In the experiments reported here the relation between velocity and hemolysin concentration was linear, but the effect of increasing hemolysin concentration was considerably less than that observed by Bernheimer. The induction period he described is also less apparent in these data and, since its length is inversely related to toxin concentration, it accounts in large part for the lower asymptotic portion of the hemolysis-log toxin relation. In addition, the data reported here show that the effects of increasing toxin and increasing red cell concentration on velocity are closely interrelated, and each is a function of the other.

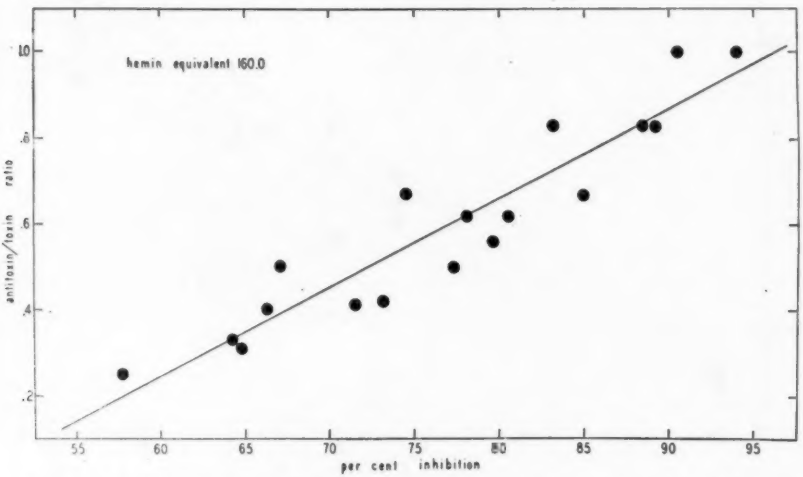

FIG. 14.—Antitoxin inhibition of hemolysis as a function of the antitoxin:toxin ratio, and measured as percent inhibition of percent hemolysis. The hemin equivalent given as 160.0 is approximate, and the extreme values were 158.2 and 167.3.

On the basis of the occurrence of an

induction period and additional evidence such as the inefficacy of antitoxin introduced late in or at the end of this period, Bernheimer (1946) has concluded that the single stage, i.e., warm phase, hemolysis produced by *Cl. septicum* hemolysin is actually a two stage reaction in which the induction period represents a primary reaction, and the secondary reaction of actual lysis is its inevitable consequence. Hemolysis by a hot-cold lysin such as α-toxin is clearly at least a two stage reaction in which lysis occurring in the cold is a consequence of changes in the red cell produced during the warm incubation phase. Since the warm phase reaction may be stopped at any point, it was possible to show that changes occurring during the induction period of hemolysis by α-toxin and arrested by chilling did not result in lysis in the cold stage, but rather that this period is preliminary to the warm phase reaction that is similarly stopped by chilling and essential to subsequent lysis.

The warm phase reaction is regarded here as the basic feature of α-toxin hemolysis, and the curious and as yet unexplained feature of its producing an increased red cell fragility apparent only on chilling is neglected in the present report. The general question, then, is that of the nature of the warm phase reaction. It is established that, in general, lysis of a given red cell is an all-or-none phenomenon (Ponder, 1948), and that partial lysis of a red cell suspension represents complete lysis of some portion of the total cells present. It is probable that more than one molecule of most hemolytic agents is responsible for lysis of the individual cell, and a constant representing the cumulative effect may be inferred from kinetic data (Ponder, 1948). The assumption that some critical number of lysin molecules combines with the red cell to produce sufficient disorientation of cell structure, so that hemoglobin is no longer bound to it, is consistent with a large body of data. In the conventional terminology of enzyme kinetics, the reaction between α-toxin (E) and the red cell substrate (S) may be represented as:

$$E+S \rightarrow E_{x-n}+E_xS+E_{x+n}S$$

when E_x is the critical concentration of toxin and E_{x-n} and E_{x+n} are sublytic and excess concentrations, respectively. The quantity represented by x is indeterminate to some degree if the percent hemolysis-toxin relation is regarded as the integral of a frequency distribution of lysin-resistance in the red cell population. At some standard incubation time, x would be relatively small for perhaps 5 to 10% of the cells and indefinitely large for another 10 to 15%, since hemolysis is asymptotic to 85 to 90%. Between these extremes it could be defined for a given percentage hemolysis corresponding, for instance, to the median or mode of the frequency distribution.

It seems unlikely that excess hemolysin inhibits the liberation of hemoglobin, and hemolysis (h) may be defined as:

$$h=F[(E_xS)+(E_{x+n}S)]$$

If the intermediate compounds arise from one another, i.e.,

$$E+S \xrightarrow{k_1} E_{x-n}S \xrightarrow{k_2} E_xS \xrightarrow{k_3} E_{x+n}S$$

the velocity of hemolysis may be a function of k_1, k_2, and k_3 together with the rates of decomposition of E_xS and $E_{x+n}S$. This differs from the Michaelis-Menton formulation in that it is assumed that the rate of decomposition of the complexes alone does not determine the velocity of hemolysis and in the postulation of a series of intermediate

complexes of which the members lacking the critical increment of lysin are not concerned directly in the complete reaction. While such application of conventional enzyme kinetics is one of the more obvious approaches to the interpretation of this reaction, the theoretical approach will be expanded along somewhat different lines in a subsequent report and need not be carried further here. The behavior of the α-toxin red cell system is consistent with this kind of formulation in that the velocity of the reaction was increased by increases in either hemolysin or red cell concentration in a linear manner. The slope of the first instance was about 0.2 and in the latter about 1.0, or an approximately 5 fold difference between the two. This has the practical consequence of apparently increasing the activity of the hemolysin on an absolute basis but decreasing it in terms of percentage hemolysis. The induction period is perhaps to be regarded as a consequence of the necessity for the reaction of a critical increment of lysin with the red cell before hemolysis can occur.

The behavior of underneutralized toxin-antitoxin mixtures indicates that the hemolysis is more complex than suggested above in that it includes at least one more variable. The slope of the linear portion of the hemolysis-log toxin relation was increased in the presence of antitoxin, i.e., the free toxin in the mixture was a more effective hemolyzing agent than the toxin alone. It is unlikely that this was a nonspecific effect of serum protein; the final mixtures contained 333 μg/ml of bovine albumin and approximately 0.6 μg/ml of serum protein in the highest antitoxin concentrations used. The relation between antitoxin concentration and the activity of the toxin measured as increase in this slope was geometric over the range of concentrations studied. This effect of antitoxin was also a function of red cell concentration, and the relation of the slope of the absolute hemolysis-log toxin relation to red cell concentration took the form of a family of parabolas for the several antitoxin concentrations (fig. 11).

It is perhaps significant that this effect was related to the red cell substrate rather than to the toxin. The slope of the linear relation of velocity to red cell concentration was essentially identical in the presence and absence of antitoxin (fig. 8), but that of the relation of velocity to toxin concentration was reduced from 0.23 for toxin alone to 0.034 in the presence of 5γ of antitoxin. In this connection the double reciprocal plot, $1/v:1/S$, showed that the relation was linear but the slope was reduced from 55.7 for the toxin alone to 31.1, and the intercept increased from 0.009 to 0.114 in the presence of 3.3γ of antitoxin. It was originally expected that these data might give some information relative to the nature of the inhibition of toxin by antitoxin, but it seems clear that more precise definition of the effect of antitoxin is required.

The observed behavior of toxin in the presence of antitoxin may be accounted for by the assumption that the toxin preparation contained an antigenic substance that inhibits its hemolytic activity. Toxoid might function as an inhibitor of toxin hemolysis, but the data reported here suggest that the inhibitor is neutralized to higher titer by antitoxin than is the toxin. It is not necessary to assume, of course, that the inhibitor is intimately related to the toxin, and if it is a contaminant it may be chemically separable from the toxin. The postulated presence of an inhibitor of hemolysis rests on more substantial grounds than inference from the observations reported here. It has been found

that, under appropriate experimental conditions, α-toxin protects against lysis of red cells by hypotonic salt solutions. For example, in hypotonic saline in which partial lysis of about 30% is produced, 1 μg of α-toxin in a volume of 3 ml gives a barely perceptible, 5% or less, lysis that is not significantly different from the lysis produced by this amount of toxin in isotonic saline. Thus the inhibitor appears to be nonspecific in that its effect is on the red cell rather than the toxin, in keeping with the observations recorded above, and its activity in very low concentrations provides the basis of a method of its titration. The inhibitor and its relation to antitoxin inhibition of hemolysis will be described in more detail in a subsequent report.

From a purely practical point of view, the linear portion of the percent hemolysis-log toxin relation between 20 and 70% hemolysis suffices to allow titration of the activity by extrapolation to an end point such as 50% hemolysis. Reproduction of the slope of fitted lines to within 5% is of the same order of accuracy as that described by Herbert (1941) in the titration of streptolysin O, and as that utilized in quantitative complement and immune hemolysin titration, and the HD_{50} of α-toxin may be taken with essentially the same degree of confidence as the 50% unit of complement or immune hemolysin.

It is essential for reproducibility that the warm phase incubation time, red cell concentration, pH, and buffer be specified as standard conditions; and that hemoglobin be determined as a stable heme compound, at least when the toxin is present as a crude filtrate. The observations reported here suggest that the reaction be carried out in acetate buffer, pH 6.0, with a 37 C incubation time of 40 minutes. Red cell concentration is preferably based on some reproducible standard such as hemin.

The titration of antitoxin by hemolysis inhibition is somewhat more complex in that the slope of the percent hemolysis-log toxin relation was found to be a function of antitoxin concentration. It must, therefore, be determined for each serum dilution, but given the slope, the HD_{50} determined by interpolation is in direct linear relation to the reciprocal of the antitoxin dilution. A hemolysis-inhibiting unit of antitoxin, defined at some convenient level, may be determined graphically, and the titer of the antitoxin read directly. Here sufficient hemolysis inhibition for accurate assay occurred in antitoxin dilutions of 1:600,000 and 1:700,000. Since the preparation contained 700 units/ml, this corresponds to the titration of as little as 1/1000 unit as the limit of sensitivity. Oakley and Warrack (1941) reported that antitoxin can be titrated on a hemolysis-inhibition basis to 1/400 unit; it seems probable that sensitivity was reduced in their titration procedure by the use of heavier red cell suspensions.

It must be emphasized that the titration procedure is based on the study of a single lot of toxin and a single antitoxin preparation. The data presented here allow no conclusions as to the reproducibility of the observed relationships on which it is based with different lots of toxin and antitoxin. Applicability in any general sense, then, requires further study directed toward that end.

SUMMARY AND CONCLUSIONS

The titration of the hemolytic activity of the α-toxin of *Clostridium welchii* and its inhibition by homologous antitoxin has been studied with respect to the development of a precise method of assay and to the relationship between toxin concentration, antitoxin concentration, red cell concentration, and the

effect of these variables on the velocity of the warm phase of the lytic reaction. It was found that the toxin was stable in dilute solution in the presence of 0.1% bovine albumin, that the optimum pH for hemolysis was 6.0, and that the buffer of choice was *M*/100 acetate. Hemolysis was measured by the spectrophotometric determination of liberated hemoglobin as ferri hemic acid and defined as its equivalent in μg of hemin. The details of titration procedure are described.

The relation between toxin concentration and percent hemolysis was described by an asymmetrical S-shaped curve with the point of inflection near the lower asymptote, and at the upper end asymptotic to 85 to 90% hemolysis. When plotted against the log of the toxin concentration, the relation was linear between 20 and 70% hemolysis. Its slope and its interpolated 50% endpoint were reproducible within about 5%. Red cell concentration was inversely related to percent hemolysis and directly related to absolute hemolysis. The velocity of the warm phase reaction, defined as μg hemin equivalent of hemoglobin liberated per minute, was directly related to both toxin and red cell concentrations; for the former the ratio of toxin to velocity was about 0.2 and for the latter that of red cell concentration to velocity about 1.0. On plotting 1/v:1/S the points fell on a straight line.

The presence of antitoxin in underneutralized toxin-antitoxin mixtures resulted in an increase in the slope of the relation of both percent and absolute hemolysis to log toxin concentration, the former becoming positive, but in a decrease in the velocity of the warm phase reaction. These data are consistent with the assumption that the toxin preparation contained an antigenic inhibitor, and that the activity of the inhibitor was neutralized by antitoxin to higher titer than the hemolytic activity of the toxin. The points 1/v:1/S plot again fell on a straight line that was decreased in slope and increased in intercept relative to that given by toxin alone. In the over-all sense, the neutralization of toxin by antitoxin occurred in multiple proportions; the relation between HD_{50} and the reciprocal of antitoxin dilution was linear, and that between antitoxin-toxin ratios and percent inhibition of percent hemolysis was also linear.

While the observations reported here are consistent with the assumption that α-toxin hemolysis is an enzyme-catalyzed reaction, the consistency can in no sense be regarded as proof that hemolysis results from enzymatic activity. On the one hand, the means by which hemoglobin is bound to the structure of the erythrocyte is far from clear. On the other hand, this lytic reaction exhibits a number of peculiarities such as the hot-cold phenomenon, the indicated critical increment of lysin per red cell and the all-or-none character of hemolysis, the possible presence of an antigenic nonspecific inhibitor active on the red cell rather than the toxin, etc. The lecithinase activity associated with the α-toxin cannot be regarded as evidence for an enzymatic basis of the hemolysis, for there has been no serious attempt to separate the two activities. Furthermore, the hydrolysis of lecithin occurs in the warm phase of the reaction only, and lecithin inhibits other hemolysins that have not been found to have lecithinase activity. In view of these and related considerations, speculation as to the nature of antitoxin inhibition of hemolysis would seem premature until the kinetics of the hemolysis itself are better understood.

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ANTIGENIC DIFFERENCES IN TWO TUBERCULIN PROTEIN FRACTIONS

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Because the question arises frequently if a positive tuberculin test may always be indicative of tuberculous infection, attempts are constantly being made to secure a product which would be reliable in this respect. The present report shows that at least two different protein fractions (here designated A and C), which can be isolated from an unheated tuberculin culture filtrate, exhibit fundamental differences in their immunological reactivities. When measured against a standard they give different relative intradermal reactions in BCG vaccinated giunea pigs from those given in guinea pigs inoculated with a more virulent strain (DT). Human beings of high and low degree of sensitivity also react differently to these two products. This difference in biological reactivity is supported by differences in chemical composition, namely the A type of protein is colorless in one percent concentration, is slower in electrophoretic mobility[1] than the C protein and different in its amino acid composition[2] and ultraviolet absorption.[1]

Antibodies elicited by the two products are not identical, as is shown below by means of the precipitin reaction and complement fixation. A difference in the antigenic capacity of these two substances has also been demonstrated in their ability to elicit the Arthus reaction i.e., with the same number of injections containing the same amount of antigen the A protein fraction caused severe Arthus reactions, with necrosis, while the C protein fraction produced much smaller reactions and never with necrosis.[3]

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MATERIALS AND METHODS

Technique.—Skin tests were made by intradermal injections with 0.1 ml of one or more unknown substances and simultaneously with 0.1 ml of a standard (PPD-S) into normal as well as guinea pigs previously vaccinated with 1 mg tubercle bacilli (BCG) or inoculated subcutaneously with 0.00025 mg of a moderately virulent strain of human tubercle bacillus (DT). In most of the series where multiple testing was done the locations of the tests were varied in order to give a completely objective aspect to the readings. Only guinea pigs which had been found on previous test with PPD-S to be good reactors were used, and any guinea pig giving doubtful or negative reactions to one fraction was excluded from the series in the final calculation. Four to 10 of such sensitized guinea pigs were tested in each series recorded in tables 2, 3 and 4, and the skin tests evident at 48 hours were measured in two diameters at right angles and the height estimated. The average measurements of each series were obtained, and then the two diameters in each case averaged. These final averages of the simultaneous reactions to the fraction and to the standard (PPD-S) are recorded, and a third column shows the differences in the sizes of these two sets of reactions. A negative difference means that the reaction to the fraction was smaller than with an equivalent dose of the standard, and a plus difference means the reverse. A similar method was used for recording the reactions in BCG vaccinated guinea pigs. Therefore, a comparison of these two columns of differences in tables 2, 3 and 4 gives a ready means of measuring potencies in animals of different

3. Seibert, F. B. 1950, J. Immunol. 65: 297–310.

sensitivities relative to a standard. A similar method was used, table 4, in comparing reactions in patients of low and high degrees of sensitivities, such as those who react to a low dose of 0.00002 mg and those who require a dose of 0.005 mg.

Fractions used.—A standard, PPD-S[4] was used in every series of tests. The C protein fractions were made by precipitating a concentrated unheated tuberculin filtrate at pH 3.8 to 4.7.[1] These fractions all exhibited a single electrophoretic component with a mobility of about -6.1 to -7.3×10^{-5} cm^2 volt^{-1} sec.$^{-1}$ In a few cases, a small additional faster component, possibly nucleo-protein, was evident. In these studies 5 such fractions, made from different strains of tubercle bacilli, as recorded in tables 2, 3, 4 and 6, were used. All were from human strains, except fraction 95 I, which was made from the BCG strain.

The A protein fractions had electrophoretic mobilities of -3.4 to -4.1×10^{-5} cm^2 volt^{-1} sec^{-1} and were all soluble at pH 3.8 to 4.7, in contrast to the C fractions. Fractions 98 III, 96 B III, 96 A IV b and 100 II from human strains were separated by alcohol fractionation at low temperature, as described earlier.[1,5] Fraction 95 II A was made from the BCG strain and showed on electrophoresis the presence of A with some other protein impurities. A protein from a bovine strain (No. 523) designated TPA, which presumably is an A type of protein because it had an electrophoretic mobility of -4.1×10^{-5} volt^{-1} sec^{-1}, was also studied.

EXPERIMENTAL RESULTS

Sensitivity of skin reaction test

Table 1 shows that when the same dose of the same material was injected simultaneously into the two arms of patients the difference in the dimensions of the reactions may be even as much as 10%. The difference in size, therefore, caused by a 20% difference in dosage (middle section of table) does not seem to be significant. Reversal of the arms made no difference.

A larger percent difference in the reaction size was found only when the dose was at least double (last section of the table). Therefore, a difference of more than 10% in the reaction size is not inconsistent with a 50 to 100% variation in the amount of material injected.

TABLE 1.—*Variation in size of reaction due to variation in PPD-S dosage or to Reversal of arms.*

No. of tests	Dose in mg	Arm tested	Average size reaction in mm*	% difference from larger reactions
Tests of humans				
44	0.00002	Left	22.2–2.7	
	0.00002	Right	19.9–2.8	10.4
26	0.00002	Left	29.6–2.6	
	0.00002	Right	30.6–2.6	3.3
166	0.005	Left	14.0–1.2	
	0.005	Right	14.3–1.3	2.1
22	0.00002	Left	32.7–2.8	
	0.000024	Right	32.6–2.7	0.3
33	0.0002	Right	32.4–2.7	
	0.00024	Left	32.2–2.7	0.6
21	0.005	Left	22.9–2.1	
	0.006	Right	22.2–2.1	3.1
6	0.005	Right	16.9–1.5	
	0.006	Left	15.6–1.5	7.6
Tests in guinea pigs				
8	0.005		19.2–2.5	
	0.0025		16.2–1.9	15.6
9	0.005		15.5–2.2	
	0.0025		13.6–1.6	12.2
8	0.002		15.8–2.5	
	0.001		12.9–1.6	18.3
	0.0005		11.5–1.5	12.1
8	0.002		13.4–2.0	
	0.001		9.6–1.3	28.3
	0.0005		8.4–1.0	12.5

* The first figures represent the average measurements of the two diameters of the reaction taken at right angles, and the second figures represent the heights.

Skin tests in guinea pigs with fractions

Skin tests in guinea pigs infected with moderately virulent tubercle bacilli (DT) (table 2) show that in 15 out of 21 series of tests, each made in 4 to 9 guinea pigs, the C type of proteins gave smaller reactions than did PPD-S, in equal dosage, based on their nitrogen contents. This difference was less if the guinea pigs had been infected with BCG than in those infected with the DT strain (compare difference columns), indicating that, relative to the PPD-S, C protein was stronger in BCG vacci-

4. Seibert, F. B. and Glenn, J. T. 1941, Am. Rev. Tuberc. **44**: 9–25.
5. Seibert, F. B., Crumb, C. and Seibert, M. V. 1950, J. Am. Chem. Soc., **72**: 2678–2684.

TABLE 2.—*Intracutaneous reactions to C proteins in tuberculous and BCG vaccinated guinea pigs.*

Type of fraction	Name of fraction and strain	In DT infected guinea pigs, average size reaction in mm*		Difference in sizes of reaction mm†	In BCG infected guinea pigs, average size reaction in mm to		Difference in sizes of reactions mm†	Dose in mg	No. of animals
		Fraction	PPD-S		Fraction	PPD-S			
C	98 I-DT	12.2−1.3	13.6−1.6	−1.4−0.3				0.0025	9
	98 I-DT	14.5−1.7	15.5−2.2	−1.0−0.5				0.005	9
	98 I-DT	17.6−2.6	17.5−2.6	+0.1−0	16.0−2.2	14.8−2.3	+1.2−0.1	0.005	7
	98 I-DT	16.7−2.4	17.8−2.7	−1.1−0.3	14.5−2.0	14.5−2.3	0 −0.3	0.005	7
	98 I-DT	11.4−1.8	13.4−2.2	−2.0−0.4				0.0025	5
	98 I-DT	9.1−1.0	10.7−1.6	−1.6−0.6				0.0025	5
	98 I-DT	8.6−1.0	11.8−1.4	−3.2−0.4				0.0025	5
	98 I-DT	12.4−2.0	14.0−2.5	−1.6−0.5				0.0025	5
	98 I-DT	13.2−1.8	16.1−2.4	−2.9−0.6				0.0025	5
	98 I-DT	16.8−2.8	15.9−2.8	+0.9−0				0.0025	5
	98 I-DT	17.9−2.2	21.0−2.7	−3.1−0.5	13.4−1.5	16.0−1.8	−2.6−0.3	0.005	6
	96 AI-A33	15.8−2.5	16.9−3.0	−1.1−0.5				0.0025	4
	96 BI-A33	15.3−2.5	14.6−2.3	+0.7+0.2				0.0025	4
	94 I-DT	14.7−1.8	13.8−2.0	+0.9−0.2	10.4−1.2‡	9.7−1.0‡	+0.7+0.2	0.005	6
	1001-H 37	13.2−2.0	15.5−2.0	−2.3−0	9.8−1.0	11.1−1.2	−1.3−0.2	0.0025	6
	1001-H 37	14.9−2.2	16.1−1.8	−1.2−0.4	9.9−1.2	11.0−1.2	−1.1−0	0.0025	6
	1001-H 37	16.6−2.6	21.1−3.3	−4.5−0.7				0.002	7
	1001-H 37	17.6−2.3	19.0−2.8	−1.4−0.5				0.002	6
	95I-BCG	14.3−2.0	13.6−1.8	+0.7+0.2	15.3−2.3	15.7−2.0	−0.4+0.3	0.005	5
	95I-BCG	14.7−1.7	15.2−1.7	−0.5−0	12.5−1.6	11.6−1.4	+0.8+0.2	0.005	6
	95I-BCG	16.5−2.2	16.1−1.8	+0.4+0.4	14.5−1.8	11.0−1.2	+3.5+0.6	0.0025	6
Average		13.8−2.0	15.8−2.2	−2.0−0.2					125
					13.0−1.6	12.9−1.6	+0.1−0		54

* See footnote to table 1.
† Differences in the average diameters (first figure) and in the heights (second figure) to the two products tested simultaneously.
‡ Five guinea pigs were used in this series.

nated pigs than in DT infected pigs.

On the other hand, reactions to the A type of protein (table 3), with rare exception (one out of 17 series), were always greater than to PPD-S. This was especially conspicuous in BCG infected guinea pigs tested with the A type of proteins isolated from the BCG or from another bovine strain of tubercle bacillus, a fact suggesting a certain degree of specificity.

Several lots of Old Tuberculin

TABLE 3.—*Intracutaneous reactions to A proteins in tuberculous and BCG vaccinated guinea pigs.*

Type of fraction	Name of fraction and strain	In DT infected guinea pigs, average size reaction in mm* to		Difference in sizes of reaction mm*	In BCG infected guinea pigs, average size reaction in mm* to		Difference in sizes of reaction	Dose in mg	No. of animals
		Fraction	PPD-S		Fraction	PPD-S			
A	98 III-DT	15.6−2.0	16.2−1.8	−0.6+0.2				0.0025	8
	98 III-DT	24.4−3.4	14.6−1.6	+9.8+1.8	19.6−2.5	10.9−1.3	+8.7+1.2	0.0025	8
	96 BIII-A33	19.8−3.0	14.6−2.3	+5.2+0.7				0.0025	4
	96 BIII-A33	17.8−2.6	14.2−2.3	+3.6+0.3				0.0025 or 0.005	13
	96AIVb-A33	19.9−3.0	16.9−3.0	+3.0−0				0.0025	4
	100 II-H37	24.7−3.5	19.0−2.8	+5.7+0.7				0.002	6
	100 II-H37	20.3−3.3	19.2−3.0	+1.1+0.3				0.002	6
	100 II-H37	18.2−2.6	14.1−2.0	+4.1+0.6	14.2−2.0	10.8−1.6	+3.4+0.4	0.0025	7
	100 II-H37	20.2−3.0	15.5−2.0	+4.7+1.0	15.2−1.8	11.1−1.2	+4.1+0.6	0.0025	6
	100 II-H37	22.7−2.8	15.7−1.8	+7.0+1.0	21.0−2.6	8.6−1.2	+12.4+1.4	0.0025	5
	100 II-H37	18.0−2.0	15.9−1.7	+2.1+0.3	17.7−2.0†	9.3−1.0†	+8.4+1.0	0.0025	6
	95 IIA-BCG	14.0−1.8	11.5−1.7	+2.5+0.1	15.9−2.2‡	9.5−1.4‡	+6.4+0.8	0.0025	6
	95 IIA-BCG	15.2−1.8	15.2−1.7	0 +0.1	18.2−2.2	11.6−1.4	+3.8+0.8	0.005	6
	95 IIA-BCG	17.5−2.0	15.7−1.8	+1.8+0.2	12.7−1.4	8.6−1.2	+4.1+0.2	0.0025	6
	TPA-BOV	19.4−2.6	17.3−2.2	+2.1+0.4	17.0−2.6§	13.6−1.9§	+3.4+0.7	0.0025	10
	TPA-BOV	14.6−1.7	14.1−2.0	+0.5−0.3	12.0−1.1	10.8−1.6	+1.2+0.5	0.0025	7
Average		18.8−2.6	15.6−2.1	+3.2+0.5					108
					15.9−2.1	10.7−1.4	+5.2+0.7		66

* See first and second footnotes to table 2.
† Five guinea pigs were used in this series.
‡ Seven guinea pigs were used in this series.
§ Nine guinea pigs were used in this series.

TABLE 4.—*Intracutaneous reactions to C proteins in tuberculous and BCG vaccinated guinea pigs.*

Type of fraction	Name of fraction dilution and strain	In DT infected guinea pigs, average size reaction in mm* to		Difference in sizes of reaction mm*	In BCG infected guinea pigs, average size reaction in mm* to		Difference in sizes of reaction mm*	Dose in mg	No. of animals
		Fraction	PPD-S		Fraction	PPD-S			
OT	Wyeth 1:400 (Lot #130H54208)	11.1–1.4†	17.3–2.2†	−6.2–0.8	10.2–1.3	13.6–1.9	−3.4–0.6	0.0025	9
	Wyeth 1:250 (Lot #130H54208)	15.2–2.1	15.8–2.4	−0.6–0.3	13.0–2.3	13.4–2.0	−0.4+0.3	0.002	8
	Wyeth 1:250 (Lot #130H54206)	14.5–2.1	15.8–2.4	−1.3–0.3	13.7–2.1	13.4–2.0	+0.3+0.1	0.002	8
	Michigan 1:40	19.1–2.8	14.5–1.8	+4.6+1.0	14.0–1.5	9.5–1.0	+4.5+0.5	0.005	6
	1:100	22.5–3.0	19.7–2.0	+2.8+1.0	11.8–1.1	11.6–1.0	+0.2+0.1	0.005	8
	1:250	20.2–2.6	20.3–3.0	−0.1–0.4	11.2–1.3	12.5–1.5	−1.3+0.2	0.005	8

* See first and second footnotes to table 2.
† Ten guinea pigs were used in this series.

(Wyeth) were tested in the same manner and the comparisons of the reactions to them and to PPD-S in guinea pigs infected with the DT or the BCG strains are given in table 4. A comparison of the two difference columns shows that less difference on the whole occurred among the BCG vaccinated guinea pigs than among the DT infected guinea pigs, indicating that these OT's were more potent in BCG infected guinea pigs, a result similar to that noted for the C protein fractions. On the other hand, another lot of OT from a different source appeared to be stronger, relative to PPD-S, in DT infected guinea pigs than in BCG infected animals, even when tested in several dilutions.

Effect of multiple tests

A factor which must be considered in a study of this nature is the effect upon the size of the reactions due to multiple testing. It was shown[6] that when simultaneous tests were made with PPD-S and an A type protein, the reactions to the A were not decreased, but when made simultaneously with a C protein fraction, the reactions to the latter were decreased, from those given by the A and the C proteins when they were tested alone in the same animals.

6. Crumb, C. and Seibert, F. B. Unpublished results.

If such effects could have been eliminated in the present study, it is possible that the A protein fractions would not have seemed as much stronger and the C protein fractions would not have seemed as much weaker than the PPD-s as they were. Nevertheless, there would still be a marked difference between the two fractions, and the A fraction would give much stronger reactions than the C fraction. As a matter of fact, when tested against each other in the guinea pigs the following results were obtained:

TABLE 5.—*Intracutaneous reactions to simultaneous tests with A and C proteins in tuberculous guinea pigs.*

Dosage in mg	Fraction	Average size reaction in mm*	% difference from larger reactions
0.0025	A	18.9–2.7	
0.0025	C	15.4–1.8	18.5

* See footnote to table 1.

Skin tests in patients with fractions

Reactions to the C type of proteins (table 6) were considerably less than to an equivalent dose of the PPD-S in highly sensitive patients, who reacted to a dose of 0.00002 mg. However, in less sensitive patients requiring a dose of 0.005 mg, the differences in the reactions to the two products were not as great.

TABLE 6.—*Intracutaneous reactions to A and C proteins in patients.*

Type of fraction	Name of fraction and strain	No. tested	Average size reaction to 0.00002 mg of		Difference in sizes of reactions mm*	No. tested	Average size reaction to 0.005 mg of		Difference in sizes of reactions mm*
			Fraction mm*	PPD-S mm*			Fraction mm*	PPD-S mm*	
C	98 I-DT	18	22.5−2.0	30.6−2.2	− 8.1−0.2	11	20.7−1.8	21.9−1.8	−1.2−0
	98 I-DT	24	23.5−2.3	34.7−2.9	−11.2−0.6	10	23.7−2.1	30.7−2.5	−7.0−0.4
	98 I-DT	42	17.7−1.8	32.7−2.7	−15.0−0.9	108	19.7−1.9	19.7−1.8	0 +0.1
	94 I-DT	22	24.8−2.4	27.6−2.4	− 2.8−0	10	17.9−1.9	17.9−1.9	0 −0
	100 I-H37	16	16.7−2.1	24.3−2.5	− 7.6−0.4	7	13.5−1.4	16.4−1.9	−2.9−0.5
	95 I-BCG	24	20.8−2.0	28.6−2.5	− 7.8−0.5	12	20.3−2.3	20.8−2.1	−0.5+0.2
	95 I-BCG	21	17.5−2.0	25.0−2.4	− 7.5−0.4	5	25.4−2.4	29.2−2.4	−3.8−0
A	98 III-DT	19	24.7−2.5	24.8−2.4	− 0.1+0.1	13	23.9−2.4	21.4−2.2	+2.5−0.2
	96 BIII-A33	15	33.0−2.6	29.4−2.5	+ 3.6+0.1	7	34.4−2.9	28.6−0.29	+5.8−0
	96 BIII-A33	50	22.1−2.2	30.6−2.5	− 8.5−0.3	91	18.3−1.7	15.2−1.5	+3.1+0.2
	96 BIII-A33	53	14.9−2.1	18.5−2.4	− 3.6−0.3	147	19.6−1.5	14.1−1.2	+5.5+0.3
	100 II-H37	29	22.8−2.2	23.4−2.2	− 0.6−0	10	19.2−2.0	18.6−1.8	+0.6+0.2
	95 IIA-BCG	18	30.2−2.6	28.5−2.6	+ 1.7−0	3	23.0−2.7	21.5−2.7	+1.5−0
	95 IIA-BCG	19	27.8−2.7	29.8−2.7	− 2.0−0	10	23.7−2.1	25.8−2.2	−2.1−0.1

* See first and second footnotes to table 2.

The reactions to the A type of protein were also usually less than to the PPD-S in highly sensitive patients (see table 6), in contrast to the reactions with the same fractions in guinea pigs. The largest decreases, however, occurred with the fraction 96 B III when it was used in the two larger series in which there were only high school children. All other groups consisted mostly of adults. In less sensitive patients the A type protein was usually definitely more potent as in the guinea pigs. The reason for an occasional discrepant series is being sought.

Moreover, it is of interest that in the two large series of subjects tested with the A protein (96 B III), in spite of the fact that smaller reactions occurred with the 0.00002 mg dose of the A protein than with the PPD-S, more actual reactors to the former were found, as shown in table 7. The average size reactions recorded in table 6 included only those who reacted to both substances.

It was also true that the variations in individual reactions between the A fractions and the PPD-S covered a much wider range than did the variations between the C proteins and the PPD-S.

TABLE 7.—*Comparison of number of reactors to protein A and PPD-S in two series of subjects.*

A type protein	No. of subjects tested	No. of positive to fraction	No. of positive to PPD-S
96 B III-A 33	276	70	62
96 B III-A 33	362	75	57

Precipitin tests

Table 8 shows that highest titers were obtained with the A antigens in the case of 4 rabbits sensitized with that antigen. Similarly the serums of 4 rabbits vaccinated with BCG gave considerably higher titers with the A than with the C antigens.

The C antiserums were not so consistent in giving the highest titers with the C antigens, although there was a decided tendency in this direction. It was notable that, in one case of C antiserum, although the titer to A antigen was 500,000, the amount of precipitate was very slight.

Complement fixation

These tests were kindly done for us by Dr. T. N. Harris, to whom we are grateful. The complement fixation titers reported by him in the last column of table 8 furnish evidence that the A and C protein antigens are different and that

the C protein appears to be the better antigen, regardless of the antiserum, in that higher titers were given by it in all three sets of rabbits. He further pointed out that the fact that a minimal optimal antigen concentration of 1:25,600 was found for the A antigen and a concentration of 1:12,800 for the C antigen, is further evidence that they differ antigenically. However, no definite antigenic specificity was noted as in the case of the precipitin reactions.

DISCUSSION

The results indicate that the A and C types of tuberculin proteins as studied differ in their antigenic capacities, and it is reasonable to believe that these differences may become greater with further purification of the antigens. For this reason it was desirable to assemble these data at this point. Furthermore, in view of the large number of possible antigens which can be isolated from tuberculin, preliminary studies on some of the cruder fractions are necessary as a directive for locating the desired antigens.

It is clear that the A type of protein, made from 6 different strains of tubercle bacilli and each tested in several series of guinea pigs, gave greater intracutaneous reactions per mg of nitrogen than did the standard PPD-S. This was true in both guinea pigs infected with virulent tubercle bacilli (DT) and in those vaccinated with BCG.

On the other hand, the C protein made from 6 different antigens in several series of guinea pigs each, gave on the average smaller reactions than did PPD-S, but the difference was not so great in BCG vaccinated guinea pigs as in those inoculated with the DT strain.

Results similar to those with the C protein fractions were noted with OT preparations of Wyeth, and this is of interest in view of the fact that the

TABLE 8.—*Precipitin titers with A and C antigens.*

Rabbit antiserums to proteins	A antigens				C antigens			Rabbit no.	Complement fixation titer to	
	98 III(DT)*	96 A IVb(A33)	96 B III(A33)	97 A(DT)	98 I(DT)	93 I(H37)	99 I(DT)		A antigen	C antigen
A+B (DT)	2,000,000 + 500,000				200,000 100,000	200,000 200,000		20 24	4	<4
		4,000,000 4,000,000	2,000,000 500,000	1,000,000 500,000			100,000 100,000	7 15 12	16	48
								1	32	>128
C (DT)	500,000† 20,000†				200,000 200,000	200,000 500,000		31 32	32 32	>128 >128
		1,000,000 10,000	500,000 20,000	500,000 20,000			1,000,000 + 200,000	14 22		
BCG	500,000 2,000,000 +				50,000 20,000	20,000 20,000		4 5	12	96
		1,000,000 2,000,000	1,000,000 1,000,000	1,000,000 500,000			10,000 10,000	9 30	32	96
								142 177	32 4	192 24

Normal serums gave negative reactions.
* The strain from which each antigen was derived is designated in parentheses following the fraction number.
† Very small amount of precipitate occurred.

chief protein apparently present in OT has a mobility and other properties similar to those of the C proteins. However, another lot of OT from a different source did not give similar results.

The series of studies in human beings here reported showed that the A antigen, while it gave smaller reactions in some series among the more sensitive patients than did the PPD-S, nevertheless picked up more reactors, and gave considerably larger reactions in the less sensitive individuals. The C protein gave very much smaller reactions than PPD-S in the more sensitive individuals but nearly equal reactions in the less sensitive ones. It would appear, therefore, that the less sensitive patients were reacting in a manner similar to the BCG-vaccinated guinea pigs.

These results suggest that studies in BCG vaccinated individuals might be helpful in selecting the most desirable antigen for demonstrating BCG sensitization and also possibly for differentiating such infection from infection with virulent organisms.

The precipitin reactions indicate that the specificities of the antigens are different and can be found in this way. They suggest, furthermore, that the A antigen may be of importance in BCG infection, in spite of the fact that it may not give rise to as large a quantity of antibodies as does the C antigen, as shown by the complement fixation tests. However, it had been proved in earlier studies to be a far better antigen in eliciting the Arthus reaction by repeated intracutaneous injection than was the C type of protein.

Since considerable polysaccharide is present in the A antigens, as so far prepared, the possibility that it plays a part should be considered. Final conclusions must wait for the isolation of A type of protein free of polysaccharide. However, the fraction 96 AIVB contained much less polysaccharide than did the other A fractions, and yet it proved to have antigenic capacities equivalent to them.

SUMMARY

Studies have been made in regard to skin reactions and antibody production to two tuberculin protein antigens (A and C) which have widely different chemical properties.

The A antigen made from several strains of human tubercle bacilli, as well as from bovine strains, including BCG, gave larger skin reactions in infected guinea pigs per mg of nitrogen than did standard PPD-S, while the C antigens from similar strains gave smaller reactions.

In general, smaller reactions occurred to all products, except perhaps proteins from the BCG strain, in BCG vaccinated animals than in those infected with a virulent strain of tubercle bacillus (DT). On the other hand, reactions to the fractions were usually larger in comparison with those to PPD-S in the BCG vaccinated animals.

In human beings the A and C fractions were weaker in the more sensitive individuals, relative to PPD-S, but both, and especially the A, were stronger in the less sensitive individuals indicating that the latter were reacting in a manner similar to BCG infected guinea pigs.

The C fraction appears to be the stronger antigen by complement fixation, but the antigenic specificity of the A and C fractions can be better recognized by means of precipitin reactions. Both tests indicate that the two types of fractions are different antigenically.

EXPERIMENTAL HUMAN SALMONELLOSIS

IV. PATHOGENICITY OF STRAINS OF *SALMONELLA PULLORUM* OBTAINED FROM SPRAY-DRIED WHOLE EGG

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Studies of the pathogenicity for man of strains of *Salmonella meleagridis*, *Salmonella anatum*, *Salmonella newport*, *Salmonella derby*, and *Salmonella bareilly* derived from spray-dried whole egg have been previously reported.[1,2,3] The present paper reports similar studies with *Salmonella pullorum*.

MATERIALS AND METHODS

The materials and methods were those used throughout the entire study and were described in detail in the first paper of the series.[1] Briefly, graduated dosages of the strains under study were fed to human volunteers following which the subjects were closely studied.

S. pullorum, strains I, II, and III were strains which had been isolated from market samples of spray-dried whole egg (4% to 6% moisture content) by the Bureau of Agricultural and Industrial Chemistry, U. S. Department of Agriculture. *S. pullorum*, strain IV, was obtained from a case of human salmonellosis.[4]

RESULTS

The results of feeding 4 strains of *S. pullorum* to human volunteer groups are summarized in table 1. The data for the noninfective dosage levels are omitted from this table but are discussed below under the respective strains. Detailed descriptions of all cases of clinical illness produced by feeding the various strains are given in the section on Description of Clinical Cases.

S. pullorum, strain I, was fed to groups of 6 men each at 8 dosage levels from 10,000 to 1,795,000,000 organisms without producing symptoms or signs in any of the subjects and with uniformly negative results from repeated stool cultures. This strain was then fed at a dosage level of 16,000,000,000 organisms. In this group every man became ill. The organism was recovered from the stools of only three of these cases. The first culture was obtained 48 hours after feeding. In the light of the data obtained from subsequent feedings (table 1), the recovery rate would presumably have been higher if cultures had been obtained 24 hours after feeding. In 4 of the cases the agglutination titer rose two dilutions or more; in one of these, from negative to 1:320. Strain

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I was subsequently fed at a dosage level of 10,000,000,000. Here again all of the men became ill. The organism was recovered from all subjects, but it will be noted that in 4 of the 6, positive stool cultures were obtained 24 hours after exhibited a rise in agglutinins.

Strain III was fed at dosages of 2,305,000; 93,500,000; and 1,295,000,000 organisms without producing illness or yielding recovery of the organism from the stool. When fed at a dosage of

TABLE 1.—*Experimental feeding of 4 strains of* S. pullorum *to human volunteers.*

Feeding no.	No. of previous feedings	No. of organisms fed	Aggl. titer		Fecal culture	
			initial	maximal	No. of times isolated	Day last recovered
			Strain I			
*350	3	16,000,000,000	1:80	1:160	1	2
*351	3	16,000,000,000	Neg.	1:80	0	0
*352	1	16,000,000,000	1:80	1:80	0	0
*353	0	16,000,000,000	Neg.	1:80	1	2
*354	0	16,000,000,000	Neg.	1:320	1	2
*355	0	16,000,000,000	1:40	1:160	0	0
*356	0	10,000,000,000	Neg.	1:80	2	2
*357	0	10,000,000,000	Neg.	1:80	1	1
*358	0	10,000,000,000	1:20	1:80	2	2
*359	0	10,000,000,000	Neg.	1:160	1	1
*360	0	10,000,000,000	Neg.	1:80	1	1
*361	0	10,000,000,000	Neg.	1:80	1	1
			Strain II			
*374	0	6,750,000,000	1:80	1:320	2	2
*375	0	6,750,000,000	1:40	1:40	1	2
376	0	6,750,000,000	Neg.	Neg.	0	0
*377	0	6,750,000,000	1:40	1:150	1	1
*378	0	6,750,000,000	Neg.	1:40	1	1
			Strain III			
*397	0	7,640,000,000	1:160	1:320	1	2
*398	0	7,640,000,000	1:80	1:320	2	2
*399	0	7,640,000,000	1:160	1:160	2	2
*400	0	7,640,000,000	1:40	1:160	2	2
*401	0	7,640,000,000	1:80	1:320	1	2
*402	2	7,640,000,000	1:160	1:160	1	1
			Strain IV			
*421	3	1,280,000,000	1:20	1:80	0	0
*422	1	1,280,000,000	Neg.	1:80	0	0
423	1	1,280,000,000	1:40	1:160	0	0
424	0	1,280,000,000	1:40	1:40	0	0
*425	0	1,280,000,000	1:20	1:80	1	2
426	0	1,280,000,000	1:40	1:80	0	0
*427	0	3,975,000,000	1:80	1:320	1	1
428	0	3,975,000,000	1:80	1:80	0	0
429	0	3,975,000,000	1:40	1:160	1	1
430	0	3,975,000,000	1:80	1:160	0	0
*431	1	3,975,000,000	1:40	1:160	1	1
432	1	3,975,000,000	1:80	1:80	0	0

* Clinical illness described under section on DESCRIPTION OF CLINICAL CASES.

feeding but not thereafter. All 6 men showed a rise in agglutination titer.

Strain II was fed at dosages of 1,387,500 and 163,250,000 organisms without production of symptoms or signs referable to the feedings and without yielding positive stool cultures. When fed at a dosage of 6,750,000,000 organisms, 4 out of 5 men became ill. The organism was recovered from the stools of each. Three of these cases

7,640,000,000 organisms, all 6 of the men became ill. The organism was recovered from the stool in each instance. Three of the men had a rise in agglutination titer of two dilutions.

When it became apparent that the three strains of *S. pullorum* obtained from spray-dried eggs did not produce illness until markedly higher doses were used than with any of the species previously reported,[1,2,3] a strain of *S. pul-*

lorum which had been isolated from a case of human illness was obtained and fed for comparison. This strain is designated strain IV. It was fed at dosages of 1,880,000; 13,900,000; and 110,000,000 organisms without giving rise to illness in any subject and without recovery of the organism from the stool. When fed at a dosage of 1,280,000,000, three of the 6 men became ill. The organism was recovered from the stool of only one, but the first cultures were not obtained until 48 hours after feeding. There was a rise in the agglutination titer of two dilutions or more in all three of the cases, and one man who did not become ill also showed a rise of two dilutions. This strain was then fed at a level of 3,975,000,000. Two of the 6 men developed illness. The organism was recovered from the stools of each, and in addition, from one of the subjects who did not become ill. In all three instances there was a rise in the agglutination titer of two dilutions.

With all strains mentioned as fed at dosages below the infective level, and not shown in the table, no subject showed any change in agglutination titer.

The incubation period was variable. Of the 27 cases, 8 had incubation periods of 10 hours or less, 12 between 10 and 20 hours, 7 of 24 hours or more; and none of longer than 48 hours. Although one man vomited at three and a half hours, this was not considered as a specific symptom, since he was without other complaints until approximately 42 hours after the feeding.

DESCRIPTION OF CLINICAL CASES

No. 350 received 16,000,000,000 organisms of *S. pullorum*, strain I. Nine hours later he was awakened from sleep by nausea, vomiting, and a diarrhea which was virtually continuous. Two hours later he was admitted to the hospital. Almost constant vomiting and diarrhea continued for 7 hours. The temperature on admission to the hospital was 100.2 F. Shortly thereafter he suffered a severe shaking chill of 15 minutes' duration, and the temperature rose to 103 F. The temperature gradually dropped to normal 14 hours later when he again had a hard chill, and the temperature subsequently rose to 104.4 F. By the following noon, 36 hours after the onset, the temperature became normal and remained so thereafter. During the first 48 hours of illness, the patient appeared seriously ill with severe prostration, marked abdominal tenderness, cramps, persistent diarrhea, and headache. Although this acute sharp illness lasted only two days, mild abdominal cramps and a diarrhea of 4 to 5 loose stools daily continued for an additional 5 days. On the first day of illness the white blood count was 19,200 with 92% polymorphonuclears and on the following day 6500. A blood culture taken 12 hours after the onset was sterile. The organism was recovered from the stool on one occasion only, two days after feeding. The agglutination titer changed from 1:80 to 1:160.

No. 351 received 16,000,000,000 organisms of *S. pullorum*, strain I. Eight hours later he became ill with chilliness, abdominal cramps, and diarrhea. During the night he passed 10 watery stools and several more the following day. Although the bowel movements were normal thereafter, he continued to complain of excessive flatus, poor appetite, and intermittent headache for a week. The highest temperature recorded was 99.2 F. The organism was not recovered from the stool, but the first culture was not made until 48 hours after feeding. The agglutination titer rose from negative to 1:80.

No. 352 received 16,000,000,000 organisms of *S. pullorum*, strain I. Ten hours later he became ill. He was awakened from sleep at 11:00 P.M. with severe abdominal cramps and feverish sensations and had 5 watery bowel movements during the night. Two more watery stools were passed during the following day. Thereafter, bowel movements were described as normal, and he had no complaints. The temperature was first obtained 48 hours after the feeding at which time it was 99.2 F. The organism was not recovered from the stool, but the first culture was not taken until 48 hours after the feeding. There was no change in the agglutination titer.

No. 353 received 16,000,000,000 organisms of *S. pullorum*, strain I. Twenty-four hours after feeding he had borborygmus but no other complaints. On the next day he passed three watery stools. The temperature was 99.2 F and the white blood count was 7600. For several days thereafter he complained of "gas on the stomach" but had normal bowel movements. A low grade fever from 99.4 F to 99.8 F persisted for a week. The

organism was recovered from the stool on one occasion only, two days after feeding. The agglutination titer rose from negative to 1:80.

No. 354 received 16,000,000,000 organisms of *S. pullorum*, strain I. Three-and-a-half hours later, he vomited once and had a slight headache for the rest of the evening. There were no complaints on the following day, but when examined 48 hours after feeding, his temperature was 100 F, and the white blood count was 7000. The rectum contained fluid fecal matter, and he stated that he had passed three watery stools that morning. He was hospitalized for 24 hours for study. No further diarrhea occurred while in the hospital, but the temperature rose to 101.2 F in the afternoon. It was normal thereafter, and he was without complaints. The organism was recovered from the stool on one occasion only, two days after feeding. The agglutination titer rose from negative to 1:320.

No. 355 received 16,000,000,000 organisms of *S. pullorum*, strain I. Twenty-four hours later he suffered nausea following the noon meal and passed one watery stool during the afternoon. When seen 48 hours after the feeding, he had had no further bowel movements or complaints, but his temperature was 100 F. He was therefore hospitalized for 24 hours' observation. During this period he had no bowel movements, no complaints, and the highest temperature recorded was 99.4 F. The white blood count was 9100 on admission and 6700 the following day. He remained well thereafter. The organism was not recovered from the stool, but the first culture was not made until 48 hours after the feeding. The agglutination titer rose from 1:40 to 1:160.

No. 356 received 10,000,000,000 organisms of *S. pullorum*, strain I. Eight hours later there was an abrupt onset of abdominal cramping, vomiting, and diarrhea. He vomited repeatedly and passed numerous loose watery stools during the night. When he was admitted to the hospital the following morning 20 hours after feeding, his temperature was 101.8 F, and the white blood count was 5800. He appeared acutely and seriously ill. His temperature rose later in the day to 104 F but promptly subsided and was normal 24 hours after admission. After admission to the hospital there were 5 loose watery stools the first day and two the second. He continued to complain of nausea and abdominal cramping. Recovery was prompt; 4 days after the onset he felt well. The organism was recovered from the stool on the first and second days after feeding. The agglutination titer rose from negative to 1:80.

No. 357 received 10,000,000,000 organisms of *S. pullorum*, strain I. Twelve hours later he developed a continuous diarrhea and vomiting. He was admitted to the hospital 19 hours after feeding, and shortly thereafter he developed a severe chill. The temperature on admission was 100 F rising three hours later to 104 F. The white blood count was 15,300 with 81% polymorphonuclears. The patient appeared acutely ill and prostrated. He was given 1500 cc of 5% glucose and saline intravenously. During the second day of illness he had 9 watery stools and complained of cramps and abdominal soreness. The white blood count was 7800. The temperature became normal 24 hours after admission. He had two loose watery stools during the third day. Severe herpes appeared on the lips and nose. He was discharged from the hospital on the 4th day. A blood culture remained sterile. The organism was recovered from the stool on the first day of illness only. The agglutination titer rose from negative to 1:80.

No. 358 received 10,000,000,000 organisms of *S. pullorum*, strain I. Eight hours later almost continuous nausea, vomiting, and watery diarrhea commenced. He was admitted to the hospital 11 hours after feeding. His temperature at that time was 100.2 F. Vomiting and diarrhea were almost continuous for the first 9 hours of illness and then subsided. On the second day of illness there were 4 loose watery stools, and he complained mainly of nausea and abdominal soreness and cramping. The temperature during the first 24 hours of hospitalization ranged from 100.2 to 101.6 F and returned to normal with only brief slight elevations on the two succeeding days. The white blood count on the first day of illness was 14,000 with 77% polymorphonuclears; on the second day it was 13,600, and on the third day 8600. He was discharged from the hospital 4 days after the onset and thereafter had no complaints. A blood culture taken at the height of his illness remained sterile. The organism was recovered from the stool on the first and second days of illness. The agglutination titer rose from 1:20 to 1:80.

No. 359 received 10,000,000,000 organisms of *S. pullorum*, strain I. Seventeen hours later he developed diarrhea. There were 9 loose watery stools during the next 24 hours. The temperature was 99 F 24 hours after feeding and 99.4 F 48 hours after feeding. He gave no other complaints. The organism was recovered from the stool on one occasion only, 24 hours after feeding. The agglutination titer rose from negative to 1:160.

No. 360 received 10,000,000,000 organisms of *S. pullorum*, strain I. Seventeen hours later he developed nausea and diarrhea and passed three watery stools. Twenty-four hours after feeding his temperature was 100 F, and the white blood count was 14,300 with 80% polymorphonuclears. He had one additional watery stool during the next 12 hours. The temperature was elevated to 99 F on the second day after feeding. He was asymptomatic thereafter. A blood culture remained sterile. The organism was recovered from

the stool on one occasion only, 24 hours after feeding. The agglutination titer rose from negative to 1:80.

No. 361 received 10,000,000,000 organisms of *S. pullorum*, strain I. Nineteen hours later abdominal cramping and diarrhea commenced. There were 5 loose watery stools in the next 5 hours. When seen 24 hours after feeding, although his temperature was normal, he complained of extreme weakness and faintness and appeared about to collapse. He was admitted to the hospital. The white blood count was 18,100 with 84% polymorphonuclears. His temperature rose to 100.6 F 12 hours after admission and remained slightly elevated for an additional 24 hours. After admission there were 8 loose watery stools the first day and three the second. He complained of nausea and abdominal cramping. He was discharged from the hospital three days after the onset and had no complaints thereafter. A blood culture remained sterile. The organism was recovered from the stool on one occasion only, 24 hours after feeding. The agglutination titer rose from negative to 1:80.

No. 374 received 6,750,000,000 organisms of *S. pullorum*, strain II. Ten hours later abdominal cramping and diarrhea commenced and continued all night. He vomited once before breakfast. Nineteen hours after feeding he had a severe chill and was then hospitalized. His temperature was 100.6 F rising in three hours to 103.2 F. He appeared acutely ill. The temperature remained above 102 F for 12 hours, and above 100 F for one-and-a-half days. He became afebrile three days after the onset. The white blood count the first day of illness was 10,900 with 80% polymorphonuclears, the second day 7400, and the third day 4700. Abdominal cramping continued for three days, and there was moderately severe diarrhea with 10 or 12 loose watery stools per day for three days. He complained of nausea and vomited once during the second day. The abdomen was moderately tender upon palpation. He was discharged from the hospital on the 4th day after the onset. Two blood cultures remained negative. The agglutination titer rose from 1:80 to 1:320. The organism was last recovered from the stool on the second day after feeding.

No. 375 received 6,750,000,000 organisms of *S. pullorum*, strain II. Forty-eight hours later he complained of having had one loose watery stool, and his temperature was 99.6 F. There were no further complaints. The agglutination titer did not change. The organism was recovered from the stool on one occasion only, two days after feeding.

No. 377 received 6,750,000,000 organisms of *S. pullorum*, strain II. Abdominal cramping and nausea developed 12 hours later followed in 6 hours by mild diarrhea. Twenty-four hours after feeding these complaints were still present, and he further complained of extreme weakness and appeared near collapse. The temperature was 99.4 F, and the white blood count was 8700. He was hospitalized immediately. The temperature rose later in the day to 101.2 F, and low grade fever continued for another two days. There were several loose stools per day for three days, and he complained of continual nausea. He offered no complaints 4 days after the onset. A blood culture remained sterile. The agglutination titer rose from 1:40 to 1:160. The organism was recovered from the stool on the first day of illness but not thereafter.

No. 378 received 6,750,000,000 organisms of *S. pullorum*, strain II. Sixteen hours later he was awakened by the onset of diarrhea. There were 5 loose watery stools in close succession. He vomited twice and complained of headache, abdominal cramping, excessive flatus, and extreme weakness. Twenty-four hours after feeding his temperature was 101.4 F, and the white blood count was 7300 with 52% polymorphonuclears. He was hospitalized. The fever promptly subsided within 6 hours with only occasional elevations thereafter. The white blood count on the second day of illness was 4500. After the first few hours diarrhea was not prominent, there being two or three watery stools a day for three days. Three days after the onset he complained only of abdominal tenderness. A blood culture remained sterile. The agglutination titer rose from negative to 1:40. The organism was recovered from the stool on the first day of illness only.

No. 397 received 7,640,000,000 organisms of *S. pullorum*, strain III. Twenty-four hours later he had no complaints, but his temperature was 101 F. The white blood count was 10,300 with 75% polymorphonuclears. He was hospitalized for purpose of study. The fever subsided within 12 hours. There was no diarrhea or other complaint at any time. A blood culture remained sterile. The agglutination titer changed from 1:160 to 1:320. The organism was recovered from the stool on one occasion only, two days after feeding.

No. 398 received 7, 640,000,000 organisms of *S. pullorum*, strain III. Abdominal cramping commenced 8 hours later and continued all night. Eighteen hours after feeding, diarrhea developed and there were 18 loose watery stools in the next 6 hours. He vomited twice and complained of aching in his legs and eyes. His temperature was 101.8 F, and the white blood count was 14,800 with 79% polymorphonuclears. He was hospitalized. Diarrhea continued for two more days with 19 and 8 watery stools per day. The temperature remained above 101 F for 8 hours after admission and became normal at the end of 28 hours. He complained of aching of the legs and eyes, head-

ache, chilliness, and abdominal cramping and soreness. He was asymptomatic 4 days after the onset. A blood culture remained sterile. The agglutination titer rose from 1:80 to 1:320. The organism was recovered from the stool on the first and second days of illness.

No. 399 received 7,640,000,000 organisms of *S. pullorum*, strain III. Abdominal cramping and diarrhea commenced 14 hours later. There were 7 loose watery stools the first day, 3 on the second, and 3 on the third day. The only temperature elevation recorded was one of 99.2 F 24 hours after feeding. There were no other symptoms. There was no change in the agglutination titer. The organism was recovered from the stool on the first and second days of illness.

No. 400 received 7,640,000,000 organisms of *S. pullorum*, strain III. Ten hours later there was a sudden onset of abdominal cramping and diarrhea. Two hours later he vomited once. Fourteen hours after feeding he was admitted to the hospital because of severe weakness and diarrhea. The temperature was normal upon admission, and the highest subsequent reading was 99.6 F during the first day of illness. The white blood count was 12,000 with 76% polymorphonuclears. Diarrhea was severe with 11 loose watery stools in the first 12 hours of illness and continuing in lesser degree for three additional days. His main complaints aside from diarrhea were abdominal cramping and weakness. The agglutination titer rose from 1:40 to 1:160. The organism was recovered from the stool on the first and second days of illness.

No. 401 received 7,640,000,000 organisms of *S. pullorum*, strain III. Fourteen hours later abdominal cramping and diarrhea commenced. There were 6 loose watery stools in the next 4 hours and no further diarrhea thereafter. Twenty-four hours after feeding his only complaint was of feeling flushed and hot. His temperature at that time was 102.5 F, the white blood count was 20,300 with 89% polymorphonuclears, and he was hospitalized. The temperature remained at 102.2 to 102.6 F for 12 hours after admission. He then had profuse sweating and the fever subsided, becoming normal 8 hours later. He promptly recovered. In the second day of illness he developed severe bilateral herpes of the lips and nose. A blood culture remained sterile. The agglutination titer rose from 1:80 to 1:320. The organism was recovered from the stool on one occasion only, two days after feeding.

No. 402 received 7,640,000,000 organisms of *S. pullorum*, strain III. Eleven hours later he was awakened by nausea, vomiting, and diarrhea. He vomited three times and passed 5 loose watery stools during the night. When seen 24 hours after feeding, he complained of severe headache and generalized aching and severe weakness. He had refused breakfast. His temperature was 101 F, and white blood count 21,500 with 86% polymorphonuclears. He appeared to be severely ill and was hospitalized. There was no further diarrhea during the first hospital day; the succeeding day there were three loose watery stools. The temperature remained above 100 F for the first 12 hours and had returned to normal 24 hours after admission to the hospital. During the second day of illness he complained of abdominal cramps and headache but had improved greatly. Three days after the onset he felt well. A blood culture remained sterile. The agglutination titer remained unchanged. The organism was isolated from the stool on the first day of illness only.

No. 421 received 1,280,000,000 organisms of *S. pullorum*, strain IV. Fourteen hours later he was awakened by abdominal cramping and passed a loose watery stool. He did not feel ill enough to report to the hospital. During the next 12 hours there were 4 additional loose watery stools. When seen 48 hours after feeding, he complained only of abdominal soreness, and his temperature was normal. The agglutination titer rose from 1:20 to 1:80. The stool was first cultured 48 hours after feeding. The organism was not recovered then or on subsequent examination.

No. 422 received 1,280,000,000 organisms of *S. pullorum*, strain IV. Thirteen hours later he was awakened by severe abdominal cramping which continued. Eighteen hours after feeding, diarrhea commenced. There were 8 loose watery stools in the next 6 hours, and he vomited once. His temperature was then 100.4 and the white blood count was 15,400 with 80% polymorphonuclears. He was hospitalized. He complained of continued abdominal cramping, and the abdomen was moderately rigid and extremely tender upon palpation. The temperature remained slightly elevated continuously for 24 hours, and there were occasional slight elevations on the two succeeding days. Abdominal cramping continued for two days, but there was no further diarrhea. Five days after the onset he felt well. A blood culture remained sterile. The agglutination titer rose from negative to 1:80. A stool culture was not obtained until 48 hours after feeding and was negative, as were all subsequent stools examined.

No. 425 received 1,280,000,000 organisms of *S. pullorum*, strain IV. When seen 48 hours after feeding he complained of having nausea for the previous 18 hours. His temperature was 99.4 F, and the white blood count was 5100. There was no diarrhea and no further complaints. The organism was recovered from a stool specimen taken that day, 48 hours after feeding. Subsequent stools were negative. The agglutination titer rose from 1:20 to 1:80.

No. 427 received 3,975,000,000 organisms of *S.*

pullorum, strain IV. He had no complaints or diarrhea at any time. Twenty-four hours after feeding his temperature was 99.8 F and on the succeeding day 99.6 F. The organism was recovered from the stool once, 24 hours after feeding. The agglutination titer rose from 1:80 to 1:320.

No. 431 received 3,975,000,000 organisms of *S. pullorum*, strain IV. Diarrhea commenced 17 hours later accompanied by mild abdominal cramping. There were three loose watery stools in the next 6 hours. His temperature at that time was 102 F, the white blood count was 20,500 with 76% polymorphonuclears, and he was hospitalized. The temperature remained above 100 F for the next 12 hours, and all subsequent readings were normal. On the second day of illness there were two loose watery stools, and he vomited once. Three days after the onset he felt well. The organism was recovered from the stool on the first day of illness only. The agglutination titer rose from 1:40 to 1:160.

DISCUSSION

In comparison with the previously reported studies with other *Salmonella* species, the dosages of these strains of *S. pullorum* which were required to produce illness in the volunteers were markedly larger. It will be noted that the dosages of the strains derived from spray-dried egg were of the same order as those required with strain IV, which was obtained from a case of human illness.

The volunteers used in this study were inmates of a state prison. Upon admission, all inmates of this institution are routinely given prophylactic immunization against typhoid, and yearly booster shots are administered thereafter. As the subjects were mostly long term inmates, many had received this immunization repeatedly.

The high dosages of *S. pullorum* required to produce illness may possibly be explained as due to cross-protection arising from typhoid immunization against a species of similar somatic antigenic structure.

The difficulties encountered in culturing *S. pullorum* from stool specimens are well known. With all strains in dosages below the levels producing illness, the organism was not recovered from the stool of any subject, even with repeated daily culturing. *S. pullorum* seeded into stools could be recovered regularly with the techniques employed. At dosage levels yielding consistent recoveries from the stool, the organisms were present for the first day or two and not thereafter. It will be noted from table 1 that approximately half of the positive stool cultures were obtained on the first day of illness only.

The clinical picture produced by *S. pullorum* was somewhat different from that noted with the other species previously reported. The explosive onset of illness in a number of the subjects is noteworthy as well as the acute nature of most of the illnesses. The high fever and prostration on the first day of illness was striking. Although the illness was often fulminating, recovery was surprisingly prompt, most subjects recovering in two or three days. One may speculate on the possible modifying effects of the previous typhoid immunization on the nature of the illness as well as upon the size of the infective dose.

SUMMARY

Three strains of *Salmonella pullorum* derived from spray-dried whole egg and one strain obtained from a human case were fed to human volunteers in graduated dosages. Human illness was produced with all 4 strains in dosages ranging from 1.3 to 10 billion organisms. The characteristic illness was fulminating but brief. The organism was isolated from the stool on the first or second day of illness only. The organism was never recovered when fed at levels lower than those producing illness. Clinical descriptions of 27 cases of human illness are given in detail.

GROWTH AND MANOMETRIC STUDIES ON CARBOHYDRATE UTILIZATION OF *BRUCELLA*

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McCullough and Dick (1943) reported the successful growth of 7 of 8 strains of *Brucella*, representing the three species, in a simple chemically defined medium with ammonium sulphate as nitrogen source and glucose as sole carbon source in a mineral base medium containing 4 accessory growth factors. Growth was maintained through ten serial cultures. In further studies (unpublished) employing 41 additional strains, 32 (10 *Brucella abortus*, 5 *Brucella melitensis*, 17 *Brucella suis*) were capable of growing in serial transplants in this simple medium. It was further found that for some strains of *Brucella* a variety of carbohydrates, carbohydrate derivatives, and amino acids could replace glucose as sole carbon and energy source.

Limited reports by others further record the inability of certain strains of *Brucella* to grow in this basal medium with glucose as the sole carbon source. Gerhardt and Wilson (1947) reported that *Br. abortus* strain 19 would not grow in this medium. By substituting lactate for glucose and using relatively large inoculums, growth occurred. The addition of 3% glycerol further increased growth, but this latter compound would not serve as sole carbon source. More recently, Gerhardt et al (1950) described successful growth of this strain of *Br. abortus* with L-glutamic acid or L-alanine as sole nitrogen, carbon, and energy sources; L-asparagine and L-histidine were found to serve in this respect if heavy inoculums (1×10^8 cells per ml) were employed. Several workers (McCullough, W. G. et al, 1947; Gerhardt and Wilson, 1948; Rode et al, 1950) have reported the inability of certain other strains of *Brucella* to grow in this synthetic medium with glucose as sole carbon source.

Although the medium does not support the growth of all strains of *Brucella* and may not furnish the optimal nutritional requirements of any strain, it affords an extremely useful tool in physiological studies with this group of organisms. In the present report, the utilization of 9 carbohydrates as carbon and energy source by 12 strains of *Brucella* has been gauged both by their ability to continuously support growth when used as sole carbon source in this basal medium and by their ability to stimulate the oxygen uptake of non-proliferating cells.

MATERIALS AND METHODS

Cultures.—Twelve strains representing 4 each of the three species of *Brucella* were used. *Br. abortus* #1, *Br. suis* #3, and *Br. suis* #4 were stock laboratory strains originally obtained from Dr. I. F. Huddleson. The remaining 9 strains were recently isolated in the authors' laboratory from the following designated sources: *Br. abortus* #2, *Br. abortus* #3, *Br. abortus* #4 (human blood stream); *Br. melitensis* #1 and *Br. melitensis* #2 (human blood stream); *Br. melitensis* #3 and *Br. melitensis* #4 (lymph node of swine); *Br. suis* #1 (bone lesion of human) and *Br. suis* #2 (lymph node of swine). *Br. abortus* #3 and *Br. abortus* #4 were carbon dioxide requiring strains;

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all other cultures grew in a normal atmosphere. With the exception of *Br. abortus* #1 (Huddleson's strain 295, known to be of low virulence), the pathogenicity of all cultures for guinea pigs had been confirmed. The dye reactions, hydrogen sulphide production, and serological characteristics of all strains were typical of the assigned species.

Carbohydrates.—Ten percent stock solutions of the following compounds were prepared by dissolving the requisite amount of carbohydrate in redistilled water: erythritol (Delta Chemical Corp.), D-fructose and D-mannose (Difco), D-sucrose (Mallinckrodt), D-xylose and L-arabinose (Pfanstiehl), D-galactose and D-glucose (Merck), and D-trehalose (BBL). The solutions were sterilized by means of sintered glass filters, transferred to sterile screw capped tubes, and stored in the refrigerator until used.

Growth studies

Utilization of carbon compounds as determined by growth in a synthetic medium with individual carbohydrates as sole carbon source

Basal medium.—The basal medium used was that developed by McCullough and Dick (1943) and of the following composition: ammonium sulphate 0.5 g, sodium chloride 7.5 g, dipotassium phosphate 1.0 g, magnesium sulphate 0.1 g, sodium thiosulphate 0.1 g, thiamin chloride 200 μg, nicotinic acid 200 μg, calcium pantothenate 40 μg, biotin 1 μg, redistilled water to make 1,000 ml, pH adjusted to 7.0 to 7.2. This basal medium was tubed in 5 ml amounts in 18×150 mm pyrex glass test tubes and sterilized by autoclaving at 15 lb for 15 minutes. The carbon compounds, previously sterilized by filtration, were added aseptically to the basal medium in a final concentration of 0.1%.

Growth tests.—With the exception of the substitution of Trypticase Soy broth (BBL) for Tryptose broth (Difco), the method of growing the organisms for the initial seeding was essentially the same as that described by McCullough and Dick (1942). A 24-hour trypticase soy broth culture was diluted 1:200 in buffered saline. The tubes of mediums were inoculated with 0.05 ml of this dilution and incubated at 37 C in the appropriate atmosphere. (The two carbon dioxide-requiring strains were incubated in closed glass containers in the presence of 10% added CO_2). Control series were inoculated simultaneously: (1) the McCullough-Dick basal medium without carbohydrate, and (2) trypticase soy broth as a check on viability and proper atmospheric conditions. The tubes were examined daily for turbidity. When definite growth was apparent (usually by the 4th or 5th day), subcultures were made into tubes of fresh medium containing the same carbohydrate. Tubes which remained clear or showed only a slight haziness were incubated for 14 days; at this time, subcultures were made into the identical synthetic medium and also into trypticase soy broth. Growth usually appeared in the trypticase soy broth within 36 hours; the other tube was incubated further, either for 14 days before discarding or until definite turbidity was observed, at which time a transplant into the synthetic medium was made. When growth on subculture was obtained, serial transplants were continued for 10 times.

Manometric studies

Utilization of carbon compounds as determined by increased oxygen consumption by washed cell suspensions

Cell suspensions.—The 48 hour growth of cells on beef liver infusion agar was suspended in 1 ml of trypticase soy broth and used to inoculate Kolle flasks of liver agar. The seeded flasks were incubated at 37 C for 36 hours and the resultant growth washed off in 20 ml of 0.05*M* phosphate buffer, pH 6.8. After filtering through sterile cotton and gauze, the cell suspension was washed by centrifuging and resuspending in fresh buffer. This procedure was repeated twice more. After the third centrifugation, the cell suspension was diluted in the necessary volume of phosphate buffer to a standard density as measured by a Klett-Summerson photoelectric colorimeter. The density used was that which when the suspension was diluted 1:10 gave a reading of 100 with filter #54 (500 to 570 mμ). This density corresponded to approximately 2 mg/ml dry weight of cells and contained, as determined by plate counts, between 25 and 30 billion viable cells/ml. The standard suspension of cells was refrigerated at 4 to 6 C prior to use.

Carbon compounds—Ten percent aqueous solutions of the same 9 carbohydrates used in the growth studies were prepared in glass-distilled water and sterilized by filtration through sintered glass filters.

Test procedure.—Oxygen uptake was determined manometrically in a standard Warburg apparatus. Warburg vessels of approximately 15 ml capacity, with a single side arm and center inner well, received 0.9 ml of phosphate buffer, pH 6.8, in the main compartment. To the side arm was added 0.1 ml (10 mg) of the carbohydrate to be tested; the inner well received 0.2 ml of 20% potassium hydroxide. The control for endogenous respiration received 0.1 ml of distilled water in the side arm. One ml of cell sus-

pension was added to the main compartment of each flask immediately prior to placing of the flasks in the water bath. All determinations were made in an atmosphere of air at 37 C. The rate of shaking was adjusted to afford 120 strokes per minute. After a 30-minute equilibration period, the side-arm contents were tipped in and manometric readings made at 15-minute intervals over a one-hour observation period. Following the completion of each test run, the reaction of every vessel was checked by the addition of brom thymol blue indicator to insure that the potassium hydroxide solution had not spilled over into the main compartment during the run. The same suspension of cells of each of the 12 strains was used in repeat runs with each of the 9 carbohydrates. The results recorded are the average obtained on two or more runs. The time interval between repeat runs did not exceed 72 hours, during which time the cell suspension was kept in the refrigerator. Refrigerated cells proved fairly stable and did not show appreciable loss of activity when refrigerated for 5 to 7 days. In most instances, the repeat runs yielded values a little lower than those obtained on the first run; this decrease in activity, however, appeared to be general and of the same degree for all substrates tested.

RESULTS

Growth studies.—Trypticase soy broth supported growth of all strains from the initial inoculum as indicated by definite turbidity within 36 hours. The initial inoculum into tubes containing the synthetic medium with the various carbohydrates developed visible turbidity at various time intervals, depending both on the strain employed and on the individual carbohydrate.

As a rule, growth in the initial transplant was characterized by a lag of from 3 to 7 days, which in the second and succeeding subcultures was reduced to a steady and constant two to three-day interval. With regard to species, the *Br. suis* cultures exhibited the shortest lag and the *Br. abortus* strains the longest, with the strains of *Br. melitensis* occupying an intermediate position.

TABLE 1.—*Growth of* Brucella *in a synthetic medium with single carbon source.*

Organism	Supplement to basal medium (0.1%)								
	Erythritol	L-Arabinose	D-Xylose	D-Glucose	D-Galactose	D-Mannose	D-Fructose	D-Sucrose	D-Trehalose
Br. abortus #1	+	(+)	+	+	+	−	+	−	−
#2	+	−	−	+	+	−	−	−	−
#3*	+	−	−	−	−	−	−	−	−
#4*	+	−	−	+	−	−	−	−	−
Br. melitensis #1	+	+	+	+	+	+	+	−	−
#2	+	+	(+)	+	+	−	+	−	−
#3	+	−	(+)	+	(+)	+	+	+	+
#4	+	−	+	+	(+)	+	+	+	+
Br. suis #1	+	+	(+)	+	+	+	(+)	+	+
#2	+	+	+	+	+	+	+	+	+
#3	+	+	+	+	+	+	+	+	+
#4	+	+	+	+	+	+	+	+	+

+ = Supported growth serially through 10 subcultures.
(+) = Initial growth which was not sustained in subculture.
− = No growth.
* Incubated in the presence of 10% added carbon dioxide.

As indicated in table 1, the combination of 12 strains and 9 carbohydrates yielded 69 instances of successful growth maintained through 10 serial transplants. Erythritol was the only compound which supported continuous growth of all 12 strains. Glucose supported serial growth of all strains except the two initially carbon dioxide-sensitive strains of *Br. abortus*. By doubling the inoculum size, *Br. abortus* #4 was grown successfully through the transfer series in glucose and erythritol but during the process, lost its requirement for an increased carbon dioxide tension. A twofold increase in inoculum size did not alter the inability of *Br. abortus* #3 to grow in glucose. This strain, however, was carried through 10 subcultures in eryth-

ritol and maintained its carbon dioxide sensitivity. In attaining a serial growth through 10 subcultures, the organisms were maintained continuously in the synthetic medium for 5 to 7 weeks. The possibility of alteration involving loss of virulence was investigated by inoculating cell suspensions of the final subculture into guinea pigs. Simultaneously, subcultures of all broths were made to plates of trypticase soy agar. Typical S type colonies which agglutinated to full titer in brucella antiserum were recovered from each plate.

With the exception of *Br. abortus* #1, the low virulence of which was previously noted, the guinea pig pathogenicity of all strains after serial passage in the different mediums was verified by development of high agglutinin titers, multiple macroscopic lesions of the spleen and liver, and recovery of *Brucella* from cultures made of heart's blood, liver, and spleen. Dye differentiation studies, hydrogen sulfide production, and serological analysis of the fresh isolates were made and in all instances were found to conform to the species characteristics possessed by the strains at the beginning of the growth studies.

Manometric studies.—The effect of 9 carbohydrates on oxygen uptake of 12 strains of *Brucella* is recorded in table 2. The values listed for each carbohydrate represent the actual results obtained. The control or endogenous respiration readings are recorded for comparison. From these data, it may be seen that there is considerable strain variation in regard to the influence of a carbohydrate substrate on oxygen uptake of resting cells. This variation is illustrated in the *Br. abortus* cultures examined with glucose, galactose, and fructose as substrate.

With few exceptions, demonstrable increased oxygen uptake was found in all of the experiments. Where there are exceptions with a particular strain, other strains of the same species yielded an increased uptake in the presence of the same compound. With *Br. abortus*, erythritol, glucose, galactose, and fructose provided superior activity. With *Br. melitensis*, glucose alone gave superior results with galactose, mannose, and fructose providing for moderate activity. With *Br. suis*, erythritol, arabinose, glucose, xylose, and galactose were most readily utilized.

The *Br. suis* strains in general gave greater activity on a larger number of compounds than the other species.

TABLE 2.—*Oxygen consumption of* Brucella *in the presence of different carbon compounds.*

Organism	Erythritol	L-Arabinose	D-Xylose	D-Glucose	D-Galactose	D-Mannose	D-Fructose	D-Sucrose	D-Trehalose	Endogenous
	cmm O_2	cmm O_2	cmm O_2	cmm O_2	cmm O_2	cmm O_2	cmm O_2	cmm O_2	cmm O_2	cmm O_2
Br. abortus #1	135.0	41.7	40.8	156.7	161.7	53.1	129.4	35.6	40.9	28.6
#2	145.0	35.9	46.5	79.1	79.0	30.0	58.9	31.1	36.0	25.6
#3	153.5	39.5	44.9	78.4	74.0	35.7	59.3	34.8	32.8	26.1
#4	146.4	38.9	61.2	113.6	80.5	39.5	101.5	37.7	33.4	24.4
Br. melitensis #1	59.2	19.2	22.4	90.8	81.0	63.0	87.0	21.0	35.2	21.0
#2	69.1	24.2	32.3	75.9	68.4	27.0	76.8	31.5	29.2	27.0
#3	51.2	34.9	33.0	124.0	48.0	81.0	78.0	27.8	55.7	26.3
#4	48.4	23.3	30.4	106.4	43.4	59.2	60.3	19.3	22.3	10.3
Br. suis #1	124.5	92.7	76.8	84.4	54.0	51.0	49.3	19.5	48.0	18.0
#2	89.4	144.4	74.3	98.5	81.3	50.2	53.3	23.2	32.8	16.0
#3	122.9	141.1	93.9	104.7	81.3	62.0	58.5	28.9	37.9	17.4
#4	101.0	97.5	69.8	86.8	70.7	50.9	40.4	38.6	47.7	12.1

cmm O_2 = Microliters of oxygen consumed during first 60 minutes observation. Flask contents: 1 ml cell suspension, 10 mg carbon substrate, 0.9 ml 0.05*M* phosphate buffer, pH 6.8.

DISCUSSION

The biochemistry of pathogenic bacteria has been studied much less than that of certain nonpathogens. With the increasing array of inhibitory substances therapeutically useful against pathogenic bacteria, this field is assuming increasing importance. Thorough understanding of the mechanisms involved in the action of antibiotics on bacteria can only be obtained after the metabolic mechanisms are thoroughly understood. Elaboration of our knowledge of the enzymatic activity of such organisms is likewise of value in the field of bacterial nutrition and pathogenesis. The present study extends our knowledge of the ability of *Brucella* to utilize certain compounds as sole source of carbon and/or energy. In the growth studies reported here with the strains employed, erythritol furnished these requirements for all 12 strains, being utilized by more strains than any of the carbohydrates mentioned. Growth with this compound in general occurred earlier than with the other substances. It is of interest that a carbon dioxide-requiring strain of *Br. abortus* was carried for 10 transplants with this compound as sole carbon source and not only grew successfully but maintained its carbon dioxide requirement throughout the 10 transfers. Strain variation in ability to utilize the carbohydrates was to be expected and has an important bearing on the problem of cultivation of *Brucella*. It is noteworthy that strains of *Brucella* can be carried in a basal medium with a variety of carbohydrates serving as sole carbon source and after numerous transfers still retain the characteristics present prior to growth in this simple medium. Further studies are needed on the role of nutrition in dissociation of *Brucella*, in the maintenance of species characteristics, and of virulence. With the strains employed, *Br. suis* appeared to possess a greater complement of enzymatic potentials then *Br. abortus*. This may help explain the greater difficulty encountered in growth of *Br. abortus* in culture mediums. In comparing results of the growth tests with those obtained in the manometric studies, correlation is not absolute. An example is the ability of *Br. suis* and two strains of *Br. melitensis* to grow successfully in sucrose. Yet in the manometric studies oxygen uptake by these strains was not marked and with certain of them was less than that obtained with *Br. abortus* strains which could not utilize sucrose for growth. The reverse of this situation is found in other instances. Inasmuch as oxygen uptake of most of the strains was stimulated to some degree at least by all compounds, one may assume that each strain possessed enzymatic potentialities for all of them. A lack of ability to grow in certain of them suggests either that such potentiality is not developed sufficiently to allow of rapid utilization or that the number of cells in a given culture with such capability was relatively small. In growth studies, opportunity is afforded for selective growth and for mobilization and adaptation of the enzymatic complex. Certain compounds may of course be utilized by some strains as energy source but not as sole carbon source. Obviously, growth in the basal medium with a single compound available proves utilization for both carbon and energy. Strain differences in metabolic pathways are well known among the bacteria. A certain strain may utilize a particular compound as energy source but not as sole carbon source, whereas another strain may utilize it for both.

SUMMARY

Twelve strains of *Brucella* (four each of the three species) were studied for

their ability to grow in a simple basal medium containing one of 9 carbohydrates (erythritol, arabinose, xylose, glucose, galactose, mannose, fructose, sucrose, trehalose) as sole carbon and energy source. The *Brucella suis* strains grew with all compounds as did the *Brucella melitensis* strains in most instances. *Brucella abortus* strains exhibited a narrower range of activity. All 12 strains were carried through 10 transplants in erythritol, while 11 were maintained in glucose. The same 12 strains and 9 carbohydrates were used in manometric studies. With few exceptions, oxygen uptake occurred with all strains and all compounds. Superior activity occurred with *Br. abortus* in the presence of erythritol, glucose, galactose, and fructose; with *Br. suis* in the presence of erythritol, arabinose, glucose, xylose, and galactose; and with *Br. melitensis* with glucose. Mannose and fructose provided for moderate activity of *Br. melitensis*.

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STUDIES IN THE PATHOLOGY AND PATHOGENESIS OF EXPERIMENTAL BRUCELLOSIS

III. INVESTIGATIONS PERTAINING TO THE FUNCTION OF THE SPLEEN

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Splenic enlargement in experimental brucella infections has been observed in all species of infected animals, but it has been especially prominent in those infections characterized by a favorable course for the host. This enlargement applies notably to brucellosis in guinea pigs caused by *Brucella abortus* and has been demonstrated in previously reported observations in which splenomegaly developed in all animals infected by various strains of *Br. abortus*.[1] The invariable enlargement of the spleen in guinea pigs infected by *Br. abortus* has also been intimated by Smillie,[2] Meyer et al,[3] and Smith.[4] Smillie emphasized that the spleen was constantly enlarged and congested. Meyer and his associates, in comparing infections in guinea pigs due to brucellas of various origins, made the observation that the enlargement of the spleen was most constant following the injection of organisms from bovine sources. They also stated that in infections caused by *Brucella melitensis*, the spleen rarely attained the dimensions in guinea pigs which were customarily produced by *Br. abortus*. It has also been observed that sublethal infections in the mouse, an animal remarkably resistant to large doses of all three species of brucella, are characterized by splenomegaly. Feldman and Olson[5] reported that there was enlargement of the spleen in every mouse infected with small numbers of both *Br. abortus* and *Brucella suis*, and that this was the only gross morphologic change observed. Heilman[6] depended on the consistent development of splenomegaly in mice infected with *Br. abortus* or *Br. suis* in experiments designed to measure the action of antibiotics. He found that in infected animals treated with various antibrucella agents, the reduction in size of the spleen was roughly parallel to the diminution in bacterial population in that organ.

As described elsewhere,[1] congestion of the sinusoids with blood has been the only demonstrable cause for enlargement of the spleen in guinea pigs infected with brucella. Attempts to demonstrate an increase in connective tissue elements by special stains were unsuccessful even in huge spleens removed

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from animals sacrificed after chronic infections of long duration. Splenic hyperemia may be an indication in increased functional activity, since hypermia is a well-known accompaniment of increased function of most tissues. The association of enlargement and congestion of the spleen with good defense against brucella suggested that a function concerned with resistance was in some way involved. The following experiments were undertaken to measure the significance of the spleen in resistance to experimental brucellosis. Because of the much higher ratio of splenic weight to body weight in mice, these animals were selected instead of guinea pigs for the experiment. A comparison in normal animals indicated that in guinea pigs weighing approximately 500 g the ratio was .0012, while in mice of 20 g the ratio was .0072, or 6 times that in guinea pigs. This suggested that the function of the spleen was more essential in mice.

METHODS

White Swiss mice weighing approximately 20 g were splenectomized under light ether anesthesia. An incision measuring 8 to 10 mm was made through the skin of the left flank. The spleen was then easily observed beneath the parietal peritoneum and delivered through a second small incision made at that point. The mesenteric attachments of the spleen were severed, but no sutures were needed to achieve hemostasis. Bleeding was slight and no infection ensued. One or two clips were applied to close the incision. Upon recovering from the anesthesia, the splenectomized mice behaved normally. The "splenectomy controls" received identical treatment except that the spleen was not cut from its mesentery, being left in situ. The splenectomized mice were observed for 7 days after splenectomy, and random samples of blood were examined for infections due to *Eperythrozoon coccoides* and *Bartonella muris*, but neither type of organism was recovered.

The splenectomized mice were first injected with an inoculum of brucella previously demonstrated to be the largest number of organisms capable of causing infection without killing the animals.[7] The purpose of this was to determine if the largest sublethal inoculum would become lethal after removal of the spleen. From a group of 40 splenectomized mice, 20 were inoculated on the 7th day with 100 million cells of *Br. abortus* suspended in 1.0 ml of saline. The same inoculations were made in 20 intact mice and in 20 "splenectomy controls." The 80 mice were sacrificed on the 18th day of infection. The liver of each animal was subjected to culture and histologic examination.

The experiment was then repeated in another 80 mice but the size of the inoculum was increased to 500,000 brucella cells. From previous studies it was anticipated that this inoculum would kill 25 to 35% of the animals. The purpose of this observation was to determine if the mortality rate would be increased in animals lacking a spleen. This study differed from the foregoing experiment in that the interval between splenectomy (or laparotomy) and inoculation of organisms was reduced from the 7 day period used in the first experiment, to 48 hours. The mice were observed for a period of 28 days.

In a final attempt to demonstrate a protective function of the spleen, the action of aureomycin against lethal infections was observed in splenectomized mice. Treatment of mice with one injection of 0.8 mg of aureomycin hydrochloride had been previously shown to provide excellent protection against lethal infections introduced into normal mice 24 hours after administration of the antibiotic.[7] Mice were pretreated at the time of splenectomy and then challenged with a lethal inoculum of *Br. abortus* 24 hours later. The spleen were first removed from 30 mice, and immediately thereafter 15 of the 30 were given a subcutaneous injection of 0.8 mg aureomycin hydrochloride. The same dose of aureomycin was administered subcutaneously to 15 intact animals. The 45 mice, as well as 15 additional normal animals, were then challenged by the intraperitoneal injection of 10 billion cells of *Br. abortus*, and the mortality rates were compared over a period of 10 days.

The strain of *Br. abortus* used for inoculating all mice in these experiments is that known as Lynch in this laboratory.[7]

RESULTS

In the first series of observations to determine the influence of splenectomy upon the course of a sublethal infection,

7. Braude, A. I. and Spink, W. W. 1950, J. Immunol. **65**: 185–199.

three of the 80 animals died. Two of the deaths were in "splenectomy controls". The other death occurred in the group of intact infected mice. Although there was no increase in mortality among the splenectomized mice inoculated with large sublethal doses of brucella, a striking difference was noted in the histologic appearances of the livers. In infected mice from which the spleens had not been removed, there were only a few focal granulomatous lesions in the liver, and the degree of cellular activity in the sinusoids was not striking. By contrast, innumerable large and small focal granulomata were present in many of the livers of infected mice which had been splenectomized. In addition, the sinusoids were filled with numerous, large Kupffer's cells. The combination of these two findings gave the appearance of increased cellular activity in the livers of mice which had been infected after splenectomy (see fig. 1). In the second experiment in which a larger inoculum of brucella was used, there was no increase in mortality following splenectomy.

Among animals receiving 10 billion cells of *Br. abortus*, nearly all untreated animals died, while the death rate among the two groups of animals treated with aureomycin was small and essentially the same. The results for the three experiments are summarized in table 1.

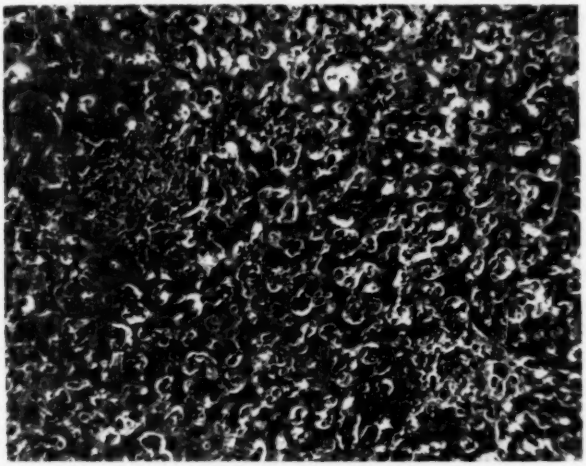

FIG. 1.—Liver of splenectomized mouse sacrificed 18 days after intraperitoneal inoculation of 100 million cells of *Br. abortus*. Note widespread formation of focal cellular aggregates in essentially every sinusoid. (Hematoxylin and eosin stain ×320)

DISCUSSION

In mice infected with sublethal numbers of *brucella* there have been no signs of ill health and no observable tissue damage of consequence.[8] In other words, the infections were well tolerated. Since these animals always presented an enlarged spleen, it appeared that additional information was availabel for correlating splenomegaly with good resistance to infection by brucella. Comparative survival rates between splenectomized and nonsplenectomized mice indicate that the spleen is not essential

for protection against the lethal action of brucella. Splenectomized mice did not succumb to large sublethal doses of brucella, and the mortality rate was not increased upon challenging splenectomized mice with inoculums which killed many nonsplenectomized mice. It was also found that the spleen was not essential for the protective action of aureomycin against lethal numbers of brucella. However, one fundamental difference was observed between splenectomized and nonsplenectomized mice and their response to brucellosis. It was observed repeatedly that there was a greater abundance of hepatic lesions in the splenectomized mice. It would appear that the spleen protected the liver against invasion by brucella. Subsequent observations in this laboratory under different experimental conditions support this conclusion.

TABLE 1.—*Failure of splenectomy to influence sublethal, lethal, and aureomycin-treated lethal infections in mice due to* Br. abortus.

Experiment	Group	Total no. of brucella cells in inoculum $\times 10^6$	Ratio of surviving mice to initial number of mice in each group
1 Sublethal infections	Splenectomy	100	20/20
	Splenectomy	0	20/20
	Splenectomy controls	100	18/20
	Intact controls	100	19/20
2 Lethal infections	Splenectomy	500	15/20
	Splenectomy	0	18/20
	Splenectomy controls	500	17/20
	Intact controls	500	13/20
3 Aureomycin-treated lethal infections	Aureomycin	10,000	11/15
	Aureomycin and splenectomy	10,000	10/15
	Splenectomy	10,000	0/15
	Intact untreated controls	10,000	1/15

It appears that the only demonstrated factor which can account for the enlargement of the spleen in experimental brucellosis is the presence in that tissue of the organisms themselves. There is some evidence, too, that the relative enlargement is proportional to the number of brucella present. Thus, administration to infected mice of aureomycin, an antibiotic which inhibits the multiplication of brucella, has resulted in a consistent reduction in size of the spleen and an associated drop in the number of organisms which could be cultivated from a cut surface of that organ. It has been demonstrated that the reduction in size of the spleen in these treated animals was not due to a specific contractile effect of aureomycin on the spleen.

Additional grounds for believing that the size of the spleen is governed by infection with brucella is offered by data from previously reported experiments.[1] In those experiments the ease of recovering brucella from the spleens of infected guinea pigs could be directly related to the weights of those organs. The correlation of the isolation of brucella from those spleens with the weights of the organ was as follows: 21 spleens weighing less than 2 g—brucella isolated from 7; 21 spleens weighing from 2 to 3 g—brucella isolated from 10; 12 spleens weighing over 3 g—brucella isolated from 10. It is true that in some animals with chronic infections there were enlarged spleens from which brucella could not be isolated. This fact does not necessarily invalidate the evidence just cited, for there is no evidence to conclude that splenomegaly subsides immediately after the organ eliminates brucella.

From present information, it would seem that splenic enlargement in brucellosis reflects the reaction of a large amount of reticulo-endothelial tissue to brucella. Upon removal of the spleen, the burden of this reaction falls upon reticulo-endothelial elements in other places. In splenectomized mice, the response to this extra burden was indicated by an exaggeration of the granulomatous reaction characteristically elicited by brucella in the liver. An indispensable defensive function could not be assigned to the spleen by experimental procedures because the reserve of reticulo-endothelium is probably great enough to cope with brucellosis in the mouse. The experiments of Marmorston,[8] on the other hand, indicate that in mice infected with the bovine tubercle bacillus or with *Eperythrozoon coccoides*, the extrasplenic reticulo-endothelium cannot fully compensate for that lost by splenectomy.

8. Perla, D. and Marmorston, J. 1935, The Spleen and Resistance, Baltimore, Williams and Wilkins.

SUMMARY AND CONCLUSIONS

1. The observation that splenomegaly was associated with good defense against brucella led to an investigation of the function of the spleen in experimental brucellosis.

2. The spleen was not found to be essential for defense against brucella, as the reticulo-endothelial elements in other organs can compensate for the loss of that tissue in splenectomized mice. However, the spleen appears to offer a considerable degree of protection to the liver against invasion by brucella.

3. The only demonstrable cause for enlargement of the spleen in experimental brucellosis was invasion of that organ by brucella.

STUDIES ON THE MECHANISM OF ANTAGONISM BY *AEROBACTER* STRAINS

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In the most detailed study to date on the antagonistic action of *Aerobacter* strains, Wynne[1] reported that one strain of *Aerobacter aerogenes* was inhibitory toward 14 of 21 common species of bacteria grown in association with the *Aerobacter* strain in broth. However, antagonism was not observed in Seitz or Berkefeld filtrates of the *Aerobacter* strain, in plates prepared from cultures heated to 60 C for one hour, nor in plates seeded with test species and massively streaked with the *Aerobacter* strain. Since no evidence was secured for the presence of a diffusible antibiotic, it was suggested that the antagonistic action noted might involve some direct action of living cells as implied in the concept of "direct antagonism" applied by Neufeld and Kuhn[2] to certain other bacteria. The present investigation represents chiefly further attempts to elucidate the mechanism of the antagonistic action of *Aerobacter* strains.

MATERIALS AND METHODS

Fourteen *Aerobacter* strains were employed. Seven were obtained from the stock culture collection of the Department of Plant Sciences of the University of Oklahoma, viz.: *A. aerogenes* 3, *A. aerogenes* 8308, *Aerobacter cloacae* 4, *A. cloacae* 5, *A. cloacae* 222, *Aerobacter mutabile* 7, *A. mutabile* 14. *Aerobacter viscosum* was obtained from the University of Nebraska. The remainder were isolated from the following sources: *A. aerogenes* 8 and 9 from human feces, *A. aerogenes* 10 and 11 from lake water, and *A. aerogenes* 12 and 16 from sewage. Morphological and biochemical characteristics of all strains were found to conform to those listed in Bergey's Manual.[3]

The species against which the *Aerobacter* strains were tested were from the stock culture collection of the University of Oklahoma, where their characteristics had been checked over a considerable period of time. They were *Bacillus subtilis*, *Bacillus mycoides*, *Bacillus polymyxa*, *Bacillus anthracis*, *Micrococcus pyogenes* var. *aureus*, *Shigella paradysenteriae* type 5, and *Salmonella paratyphi*. All 7 of these organisms were among the species found susceptible to *Aerobacter* inhibition by Wynne.[1]

For the preparation of "staled" agar, *Aerobacter* broth cultures were incubated for 3 days unless otherwise indicated and, after desired treatment, were mixed with an equal volume of double strength nutrient agar. The temperature of the two components was so adjusted before mixing that the final temperature of the "staled" agar was around 45 to 50 C, and 15 to 20 ml were poured into petri plates without undue delay. Thus the presence of living *Aerobacter* cells in the "staled" agar was insured except in those cases where prior treatments resulted in lethal effects. Each plate was inoculated shortly after solidification with two test organisms by means of single massive streaks from 24-hour broth cultures with a carefully standardized loop needle.

Nutrient broth was prepared from the Difco dehydrated product, and nutrient agar by the addition of 1.5% Difco agar to the broth. Observations were made at 24 hours of incubation, and in certain cases periodically thereafter. All incubations were at 37 C.

EXPERIMENTAL

Antagonism on solid medium.—Three-day broth cultures of the 14 *Aerobacter*

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2. Neufeld, F. and Kuhn, H. 1934, Untersuchungen ueber "direkten" Bakterienantagonismus. Ztschr. f. Hyg. u. Infektionsk. **116**: 95–110.
3. Breed, R. S., Murry, E. G. D. and Hitchens, A. P. 1948, Bergey's Manual of Determinative Bacteriology, ed. 6, Baltimore, Williams and Wilkins Co.

strains were used to prepare "staled" agar as described above. The 7 test organisms were then streaked on this agar, and all were inhibited by each of the *Aerobacter* strains, though to varying degrees (table 1). Observations at 24 and 72 hours of incubation were essentially identical, indicating that antagonism was not overcome by prolonged incubation.

Four of the most antagonistic *Aerobacter* strains were chosen for use in further tests, viz.: *A. viscosum*, *A. aerogenes* 3, *A. aerogenes* 8, and *A. aerogenes* 8308. Similarly, four species were selected as test organisms: *M. pyogenes* var. *aureus* and *S. paratyphi*, which were completely inhibited, and *B. subtilis* and *B. mycoides*, which were inhibited to a somewhat lesser degree.

TABLE 1.—*Growth of seven test organisms upon* Aerobacter *"staled" agar.*

Aerobacter strain	Test organism						
	B. subtilis	*B. mycoides*	*B. polymyxa*	*B. anthracis*	*M. aureus*	*Sh. paradysenteriae*	*S. paratyphi*
A. aerogenes 3	0	1	0	0	0	0	0
A. aerogenes 8	0	1	0	0	0	0	0
A. aerogenes 9	3	3	0	1	0	0	0
A. aerogenes 10	3	3	0	1	0	0	0
A. aerogenes 11	2	2	0	1	0	0	0
A. aerogenes 15	0	1	0	0	0	0	0
A. aerogenes 16	0	1	0	0	0	0	0
A. aerogenes 8308	0	0	0	0	0	0	0
A. cloacae 4	2	3	0	1	0	0	0
A. cloacae 5	2	3	0	2	0	0	0
A. cloacae 222	3	3	0	2	0	0	0
A. mutabile 7	0	3	0	2	0	0	0
A. mutabile 14	1	2	0	1	0	0	0
A. viscosum	1	1	0	1	0	0	0

0—Complete inhibition. 1—Pronounced inhibition. 2—Moderate inhibition. 3—Slight inhibition. 4—No inhibition, growth equal to control.

When *Aerobacter* strains were massively streaked on plates seeded with the test organisms, no inhibition zones were observed. Such zones would indicate the production of a diffusible inhibitory substance. It is, of course, conceivable that inhibition may have occurred on the surface of the agar directly beneath the *Aerobacter* growth, as pointed out by Wynne,[1] but such inhibition would not be detectable by visual observation.

Filtrates.—Seitz filtrates of 3-day broth cultures of the 4 *Aerobacter* strains were employed in the preparation of "staled" agar as above. None of the 4 test species was inhibited. To test sterility, filtrates were incubated 3 days, and inoculations were also made into broth.

A broth method of testing the filtrates was also employed. Two ml were added to an equal volume of double strength nutrient broth. Sterile water was used in place of filtrates for controls, and inoculations were made from 24-hour broth cultures of the test species. Again no inhibition was noted.

Dialysates.—The *Aerobacter* strains were grown in cellophane dialysis bags suspended in nutrient broth in 250 ml Erlenmeyer flasks. After 3 days of incubation the dialysates were tested in agar and broth by the methods used in the study of filtrates. No inhibition of any of the test species was noted. Uninoculated controls were sterile.

Heat killing.—Three-day cultures of the 4 *Aerobacter* strains were heated to 75 C for 20 minutes and used as a base for preparation of "staled" agar plates as above. Growth of the 4 test organisms on such plates was equal to and, in some cases, better than that on controls. Possibly the heat-killed cells provided added nutritive material.

Though these findings suggest that living cells may be essential to *Aero-*

bacter antagonism, a heat labile nonfilterable inhibitory substance could be involved. Therefore another method of killing was employed.

Chloroform killing.—One ml of chloroform was added to 35 ml of 3-day *Aerobacter* broth cultures, and the mixture was violently agitated for 30 seconds. The chloroform was allowed to settle, and approximately 20 ml were decanted into sterile bottles and used as a base for the preparation of "staled" agar plates as above. No inhibition of the test organisms was observed. Sterility of the supernatant from the chloroform treatment was established by inoculation into nutrient broth.

Centrifugation and washing.—In an attempt to remove diffusible metabolic products, 3-day cultures of *Aerobacter* were centrifuged at 2200 r.p.m. for 5 minutes and the cells washed 3 times in sterile water. The cells were then suspended in sterile water, an equal volume of double strength nutrient agar was added, and plates were poured as above. Inhibition of the test species on such plates was comparable to that observed on ordinary "staled" agar prepared from whole broth cultures.

Effect of time of incubation on antagonism.—If an antibiotic substance were involved in *Aerobacter* antagonism, the degree of inhibition might increase with age of the *Aerobacter* culture. Twelve- and 24-hour broth cultures were used as bases for the preparation of "staled" agar plates. Inhibition of the 4 test species was essentially comparable to that exhibited by 3-day cultures.

Dilutions of Aerobacter *cultures.*—Three-day broth cultures of 3 *Aerobacter* strains were diluted 1:10, 1:100, 1:1000, 1:10,000, 1:100,000, and 1,000,000. An equal volume of double strength nutrient agar was added and plates prepared in the usual manner. Results in table 2 show some decrease in inhibition at the 1:1000 dilutions, with relatively slight inhibition persisting in most cases at dilutions of 1:10,000.

TABLE 2.—*Growth of four test organisms upon plates prepared from dilutions of* Aerobacter *cultures.*

Dilution	*A. aerogenes* strain	Test organism			
		B. subtilis	*B. mycoides*	*M. aureus*	*S. paratyphi*
Undiluted	3	0	1	0	0
	8	0	1	0	0
	8308	0	0	0	0
1:10	3	1	1	0	0
	8	1	1	0	0
	8308	1	1	0	0
1:100	3	1	1	0	0
	8	2	2	0	0
	8308	1	1	0	0
1:1000	3	2	1	1	1
	8	3	2	2	1
	8308	1	2	1	1
1:10,000	3	3	3	3	2
	8	4	3	3	3
	8308	3	3	3	2
1:100,000	3	4	4	4	4
	8	4	4	4	4
	8308	4	4	4	4
1:1,000,000	3	4	4	4	4
	8	4	4	4	4
	8308	4	4	4	4

See table 1 for explanation of symbols.

DISCUSSION

Results of the dilution experiments may seem at first sight to indicate par-

ticipation of a diffusible inhibitory substance. However, if such were the case, it appears likely that the test organisms would exhibit rather pronounced differences in dilution effects, since organisms generally differ widely in sensitivity to antibiotic substances. Inhibition of all 4 test species became approximately the same at dilutions of 1:10,000 and ceased at dilutions of 1:100,000. Furthermore, the fact that antagonism was not observed at moderately high dilutions could conceivably result from the absence of sufficient numbers of *Aerobacter* cells in relatively close proximity to cells of the test species. The latter explanation is compatible with the occurrence of "direct antagonism."

Other findings obtained lend added feasibility to the hypothesis of "direct antagonism" being involved in *Aerobacter* antagonism. The fact that inhibition observed on plates prepared from washed *Aerobacter* suspensions occurred after the same incubation period as that required for comparable inhibition by whole cultures is difficult to explain on the basis of participation of an antibiotic substance. Again, the observation that 12-hour cultures were as antagonistic as 72-hour cultures is significant in showing that prior incubation time of the *Aerobacter* cultures did not appear to be a factor in the degree of antagonism produced. Finally, an inhibitory effect independent of living cells could not be demonstrated by any of the means employed, viz.: filtration, dialysis, thermal killing, killing with chloroform, or streaking *Aerobacter* species on plates seeded with the test organisms.

Although none of the above 7 lines of experimental work would be convincing if considered separately, it is felt that together they give considerable plausibility to the view that antagonism by *Aerobacter* strains may depend on the presence of living cells in fairly close contact with the cells of the inhibited species. Recent work on the mechanism of antagonism by strains of *Gaffkya tetragena* (Frankel and Wynne)[4] has indicated that it, too, may involve "direct antagonism" in the sense of Neufeld and Kuhn.[2]

SUMMARY

Fourteen *Aerobacter* strains were tested against 7 common bacterial species by the "staled" agar technique. Antagonism in varying degrees was noted in all cases. Further work was done with 4 of the most antagonistic *Aerobacter* strains and 4 test species.

Evidence for a diffusible inhibitory substance could not be obtained through use of *Aerobacter* culture filtrates, cellophane bag dialysates, heat-killed suspensions, chloroform-killed suspensions, or plates seeded with the test species and streaked with *Aerobacter*. Furthermore, washed cells gave inhibition equal to that obtained with whole cultures, and 12-hour cultures were equivalent to 72-hour cultures. Inhibition was noted in plates prepared from dilutions of *Aerobacter* cultures up to 1:10,000, at which dilution all 4 test species were inhibited to a similar degree.

"Direct antagonism" (Neufeld and Kuhn)[2] involving relatively close contact of living *Aerobacter* cells with cells of the inhibited species (Wynne)[1] is felt at present to be the most feasible explanation of *Aerobacter* antagonism.

4. Frankel, J. J. and Wynne, E. S. 1951, Antagonism by *Gaffkya tetragena* strains. J. Infect. Dis. **89**: 52–55.

STUDIES IN BACILLARY DYSENTERY

III. MOBILIZATION OF SPECIFIC ANTIBODY INTO THE INTESTINE OF THE RABBIT AS A RESPONSE TO HOMOLOGOUS LIVING DYSENTERY BACILLI

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The infections caused by microorganisms of the shigella group are confined to the intestines. The immune mechanisms in shigellosis are not well understood, and the significance of human antibody titers, as measured by agglutination tests, are in doubt. In recent years the role of coproantibodies in enteric infections has been the subject of several investigations. Burrows et al[1] have published the results of their studies on immunity in experimental cholera in guinea pigs. Harrison and Banvard[2] reported that coproantibody was excreted in various enteric infections in humans. Gordon et al[3] found significant agglutinin titers in feces only at one- to two-day postinoculation intervals in subjects given shigella vaccine parenterally. González et al[4] failed to detect antibodies in the feces of patients with bacillary dysentery (Flexner). Koshland and Burrows,[5] who made comparative studies between antibody titers in feces and serums, concluded that they were independent of each other. In the present investigation an attempt was made to study, in isolated segments of intestine of normal and immune rabbits, the antibody content of the lumen after the introduction of living dysentery bacilli.

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MATERIAL AND METHODS

A series of rabbits was immunized with a formalin-killed vaccine prepared from a culture of *Shigella paradysenteriae* type I so as to stimulate the development in their blood of a high agglutinin titer to the specific antigen used. Blood samples were obtained from these animals, and after measuring their antibody content, the serums were stored in the cold for use later in the experiment. The titer of the serums ranged from 1:2500 to 1:20,480.

A series of normal rabbits also was bled and the agglutinin titers for dysentery bacilli were determined.

Both normal and immune animals were operated under ether anesthesia. The abdomen was opened and the vermi orm appendix was tied with catgut size 00, isolating it completely from the caecum. Immediately afterwards, 0.5 ml of a saline suspension of a tryptose agar culture of *Sh. paradysenteriae* type I was injected into the lumen of the appendix, utilizing a fine needle, gauge 26. Some of the normal rabbits also received, simultaneously with the bacterial suspension, an injection of 1 ml of immune rabbit serum. The animals were killed at different time intervals after the operation; the appendix was removed and the fluid contained in it was examined both for agglutinating antibodies and for viable dysentery bacilli. (See tables 1, 2, and 3.)

TABLE 1.—*Immune rabbits that received injection of 0.5 ml bacterial suspension* (Shigella *type I*) *into isolated segment of intestine.*

Rabbit no.	Agglutinin serum	Time of removal of appendix after operation (in hours)	Agglutinin titer of appendical fluid	Culture from fluid for shigellas
127	1:20,480	6	1:40	+
78	1:10,240	6	0	+
43	1:10,240	16	0	+
26	1:10,240	24	0	+
80	1: 2,560	24	0	+
173	1:10,240	24	1:20	+
89	1: 2,560	24	0	+
23	1:10,240	30	1:160	+
770	1:20,480	48	1: 40	+
933	1:10,240	48	0	+
793	1:10,240	72	0	+
191	1:10,240	144	1:20	Contaminated

TABLE 2.—*Normal rabbits that received injection of 0.5 ml bacterial suspension* (Shigella *type I*) *into isolated segment of intestine.*

Rabbit no.	Agglutinin titer in serum	Time of removal of appendix after operation (in hours)	Culture from fluid for shigellas
970	0	6	+
898	1:160	16	+
955	1: 80	24	+
5	1:160	24	Contaminated
980	0	24	+
62	1:160	30	+
7	1: 80	48	+
103	1: 40	48	+
895	1: 80	72	Contaminated

The agglutinin titer of appendical fluid was negative in all cases.

TABLE 3.—*Normal rabbits that received injection of 0.5 ml bacterial suspension and 1 ml of immune serum into isolated segment of intestine.*

Rabbit no.	Agglutinin titer in serum	Agglutinin titer of immune serum injected	Time of removal of appendix after operation (in hours)	Agglutinin titer of appendical fluid	Culture from fluid for shigellas
131	1:160	1: 5,120	24	1:20	+
188	1: 20	1: 2,560	24	0	0
21	1:320	1: 5,120	48	0	+
156	1: 20	1: 5,120	48	0	+ (Fewer)
190	1: 80	1:10,240	48	0	+

RESULTS AND DISCUSSION

The appendices from all rabbits were found on removal to be greatly enlarged and showed distention almost to the point of bursting; they were hyperemic and some presented dark purplish patches. The distended lumen contained large amounts of fluid, fibrin shreds and debris. In some cases the liquid was tinted with blood or was of a brownish color, suggesting the presence of fecal matter. Sections of the organ were fixed in Zenker's fluid and prepared for histological studies. On microscopic examination all the animals showed intense congestion and more or less extensive hemorrhages into the superficial portions of the mucosa. In many of them the hemorrhages were more extensive, involving the superficial half of the mucosa and small areas in the remainder of the wall of the intestine. This was accompanied by evidences of moderate autolysis in those portions, together with dense bacterial growths in those areas secondary to autolysis. There was no inflammatory reaction whatsoever in the mucosa.

The cultures made from the contents of the intestinal segments on MacConkey's agar yielded *Sh. paradysenteriae* type I, with the exception of one case (rabbit 188), in which no growth was obtained. Four other cultures were contaminated heavily with *Escherichia coli*, so that dysentery bacilli could not be recovered. In all cases in which bacterial growth was obtained the colonies were so numerous that the estimation of the number was impossible on the original plates. Besides, the differences in the approximate numbers of colonies among the plates made from the immune and the normal animals, with or without the addition of the antiserum, was so insignificant that the actual determination of the amount of bacterial growth could not have provided evidence to clarify the objective of the experiment. However, we observed that, in general, the cultures from the normal rabbits yielded more colonies than those from the immune animals.

The agglutination reactions carried out with the fluid contents of the appendices removed from all animals were

negative, except for 5 of the 12 immune rabbits and 1 of the 5 animals injected into the lumen of the organ with antiserum.

Seven of the normal rabbits that received the injection of the bacterial suspension had "normal" agglutinin titers in their blood ranging from 1:40 to 1:160. Two did not show agglutinating antibodies. None of the fluids recovered from the intestinal segment at autopsy had agglutinins for *Sh. paradysenteriae* type I. A positive culture for this organism was obtained from this fluid from 7 of the rabbits, while 2 cultures were contaminated with *Esch. coli*.

The 5 normal animals that received 1 ml of immune serum besides the bacterial suspension had "normal" agglutinin titers in the blood ranging from 1:20 to 1:320. Only rabbit 131 had demonstrable agglutinating antibodies with a titer of 1:20 in the appendicular fluid. This animal 24 hours before the removal of the appendix had been given 1 ml of an immune serum with a titer of 1:5120, plus the bacterial suspension, by injection into the lumen. From these results, the serum antibodies disappeared within 24 hours after they were introduced into the intestines.

Of the 12 immune rabbits used for the experiment, all with high agglutinin titers in their serum, only 5 had demonstrable antibodies in the appendicular fluid, in titers ranging from 1:20 to 1:160, values that are far below those in the serum. Apparently, if the circulating antibodies permeate into the lumen of the bowel their amount is very insignificant compared with the titers in the blood serum, or else they rapidly disappear from the intestine, as evidenced by the results obtained in the normal rabbits that were purposely injected into the lumen of the appendix with antiserum of high agglutinin titers. However, the mechanism of the disappearance of these agglutinins, whether due to disentegration brought about by enzymic reaction or to absorption by the great number of homologous bacteria present in the intestines, was not determined in the present experiment.

Koshland and Burrows,[8] in their work on immunity to cholera, came to the conclusion that fecal antibody behaves independently of that of the serum, and that therefore it is not derived from the latter. In the foregoing experiment the animals were kept alive for a short period of time. In only one case was the rabbit killed 5 days after the operation, all other animals having been sacrificed at intervals ranging from 6 to 72 hours. Thus, the period during which the intestinal mucosa was exposed to the bacterial agent was relatively short, and probably not long enough for the production of antibody locally. Rabbit 127 was killed 6 hours after the injection of the bacterial suspension into the appendix and was found to have agglutinin titer of 1:40 in the appendicular fluid. The difference in agglutinin titers between the serum and the appendicular fluid could be due, as suggested above, either to enzymic destruction or to bacterial absorption.

In all the animals studied the isolated appendicular segment was distended with fluid which necessarily must have passed into the intestinal lumen from the circulation. Accepting the permeability of the mucosa to the serum antibodies, the quantity of the latter that went into the appendix must have been in proportion to the agglutinin level in the blood, which was high. The absence of antibody, or its low titer in the fluid recovered from the appendices of the immune rabbits, strengthens the assumption indicated in a previous paragraph of the rapid disappearance of antibodies from the intestinal lumen. Consequently, the difficulty of finding

agglutinins in the feces in cases of bacillary dysentery could be similarly explained. We may suggest further that the low antibody titer that may remain in the intestinal tract will be diminished to practically nil after dilution with the total contents of the bowels.

Even though dysentery is essentially a local infection of the intestinal mucosa, the process of acquired immunity will develop gradually during the course of the disease, the mechanism of which probably follows the same characteristics of general immunity, antibodies first being produced in cells or by cellular activities in the mucosa.

The length of time, as Taliaferro[6] stated in discussing immunity to intestinal parasites, that they may stay localized must depend upon their locus of formation with reference to the blood and lymph stream, their concentration, and whether or not the antigen remains localized. In this particular case the site of genesis of the antibodies may not be freely accessible to the circulation and their removal or transportation to form part of the general demonstrable immunity in the blood is perhaps delayed. Moreover, a probable factor that may also retard the appearance of the antibodies in the circulation when they have already been detected in the feces during the initiation of the infection, as pointed out by other students of the problem, is the proximity of the antigen (the dysentery bacilli), which remains localized in the intestines and may possibly attract and absorb the antibody as it is produced locally. Thompson and Olson[7] in studying the antibody production in the rabbit's cornea suggest that presumably the antibodies should be demonstrated earlier in the area of formation than elsewhere, but there is a masking effect of the residual antigen unless there is a high enough concentration of antibody to overcome this effect.

Such "interplay," as referred to by Taliaferro, between the production and local recruitment of antibodies and cells takes place in the lamina propria of the intestines until the infection is fully controlled and the number of offending bacilli diminishes. Then the antibodies, whose concentration may have increased by this time, are carried into the circulation and become detectable by the usual serologic tests. The dysenteric infection may be in its later stages by that time.

Raffel[8] states that if this mechanism of protection is the true picture in bacillary dysentery, it is not as effective as that in cholera; for in the latter disease the resistance resulting from an attack rapidly clears organisms from the intestines, while in the former the recovery is commonly followed by a carrier state of variable duration. "May this be because," says Raffel, "an antibody-complement lytic mechanism is very effective in the destruction of *V. comma*, and is much less, if of any significance in the case of the Shigellae?"

SUMMARY

Normal and immune rabbits were operated on to produce an isolated segment in their bowels. The vermiform appendix was tied and a suspension of *Shigella paradysenteriae* type I was injected in the lumen. Some of the normal rabbits received, in addition to the bacterial suspension, 1 ml of immune rabbit's serum. At different intervals of

6. Taliaferro, W. H. 1940, The mechanism of immunity to metazoan parasites. Am. J. Trop. Med. **20**: 169–182.

7. Thompson, R. and Olson, H. 1950, Antibody production in rabbit's cornea J. Immunol. **65**: 633–651.

8. Raffel, S. 1949, Types of acquired immunity against infectious disease. Ann. Rev. Microbiol. **III**: 221–264.

time the animals were killed and the contents of the appendices were examined for viable bacilli and for antibody. Agglutinins were found in low titer only in 5 of the immune rabbits and one of the normal rabbits that were injected with the immune serum. Viable dysentery bacilli were isolated from the contents of the intestinal segment of all the rabbits except one.

The low agglutinin titer in the appendicular fluid in the immune rabbits as compared to the antibody level in their blood, and the disappearance of agglutinin in the normal rabbits that were injected with immune serum, was not investigated in the present work; but we suggest that a probable explanation could be the absorption of agglutinins by the homologous bacteria in the intestines or disintegration of antibody caused by enzymic action.

STUDIES ON THE IN VIVO FATE OF BRUCELLA VARIANTS WITH DIFFERENT VIRULENCE

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Differences in colony types of *Brucella* are usually associated with differences in virulence (Braun, 1950). They therefore provide simple markers for determining the fate in vivo of variants with different virulence after simultaneous or successive inoculation of two or more variant types. It is of considerable value for various phases of research on brucellosis to utilize such marked cell types for studies in vivo. First, by adjusting infective doses of successively administered inoculums so that ID_{50}'s are comparable, it becomes possible to study certain immunologic and pathogenetic problems. Since descendants of the first inoculum can be easily distinguished from the progeny of the second inoculum by observing colony types on plates streaked with tissue samples from the infected animals, the proportion of cells recoverable from the primary or secondary infection can be ascertained rapidly in any tissue. Preliminary data, obtained by checking isolates from guinea pigs infected with homogeneous cultures of a given colony type, had indicated that the stability in vivo of the particular types tested was sufficiently great to permit their use as markers. Secondly, this technique permits investigation of certain problems associated with a chronic disease such as brucellosis. Previous in vitro studies (Braun, 1949) had shown that the less virulent nonsmooth types do not establish themselves in originally smooth cultures that are maintained in the presence of small amounts of normal serum from susceptible animals. In contrast, a rapid establishment of these less virulent nonsmooth variants was observed in vitro in the presence of serums from infected or immunized animals. These effects were shown to be independent of the presence of agglutinins. On the basis of these results it was believed that less virulent nonsmooth types may also establish themselves in vivo some time after an acute infection but may be replaced eventually by virulent types when selective conditions in the infected host return to a status analogous to that in normal susceptible hosts. It appeared that such population changes might play a role in inducing the frequently observed relapses. The findings in one extensive experiment with guinea pigs (Braun, 1950), in which an intermediate variant of *Brucella suis* gradually replaced a smooth type, seemed to substantiate this assumption. However, it was not possible to detect a similar replacement of smooth types by nonsmooth types in several subsequent guinea pig experiments.

In order to obtain further information on this point, as well as information on the efficacy of the "marker" technique which was discussed earlier, the fate of mucoid variants of *Br. suis* in guinea pigs previously infected with smooth *Br. suis* cells was investigated.

A series of unpublished animal experiments preceded the work which will be reported here. These preliminary experi-

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ments supplied information regarding dosages and routes of inoculation and, therefore, deserve a brief recapitulation here. First, 3 groups of 16 guinea pigs each were inoculated with mixtures of a virulent, smooth *Br. suis* and a less virulent, rough-like I_4 variant; the proportions of S to I_4 were (a) 99:1, (b) 50:50, or (c) 1:99. Each animal received a total of 1000 cells subcutaneously, and after infection animals were sacrificed at weekly intervals for 4 weeks. Culturing of the regional (inguinal) lymph node and the spleen revealed that no I_4 types could be isolated at any time from animals of groups A and B. Animals of group C showed a progressive establishment of the S type, and by the 3rd week after infection only the S type was isolated from the spleen of most animals. This indicated that after simultaneous infection with S and non-S types, the establishment of the more virulent type proceeded very rapidly within the first 3 weeks after infection, and that, in order to determine the fate of non-S types for prolonged periods after infection, a massive dose of the less virulent type is required. Accordingly, in a 2nd animal experiment, 45 guinea pigs were first infected with 24 cells of the S strain and then reinfected intramuscularly in the hind leg with 2×10^3 or 2×10^9 I_2 (intermediate 2) and M (mucoid) cells 6 and 13 days after the primary S infection. Up to 4 weeks after S infection, this permitted the recovery of non-S types from the spleen and regional lymph node of most animals that had received the massive dose (2×10^9) of the less virulent type. No non-S types could be recovered from the testes or bone marrow of such animals. In addition, it was noted that the percentage of recoverable non-S types in individual animals was usually much lower in the spleen than in the inguinal lymph node, suggesting the possibility of a blocking effect by the lymphatic system.

These results indicated that the fate in vivo of the less virulent non-S variants could best be determined (a) by administration of S and non-S types successively, (b) by use of massive doses of the non-S type, (c) by culturing of a variety of tissues at various intervals after infection, and (d) by utilization of two routes of administration of the non-S type, one that may involve lymphatic blocking through the use of subcutaneous reinfection and another that would minimize such blocking through the use of intracardial reinfection. The results of experiments incorporating these conditions will be described in this report.

MATERIAL AND METHODS

Guinea pigs were inoculated subcutaneously or intracardially with massive doses of a mucoid variant of *Br. suis* 1 week or 5 weeks after primary infection with smooth organisms. The intervals of 1 or 5 weeks between S and M infections were chosen because studies in vitro had shown that the selective activity of guinea pig serums was unaltered 1 week after S infection but had undergone changes 5 weeks following S infection. The smooth *Br. suis* cells were injected subcutaneously in the right inguinal region; each animal was infected with 30 cells, which represents approximately 10 ID_{50}'s. One group of animals was next inoculated 1 week later with 2 billion mucoid cells per animal *subcutaneously* near the site of the primary infection; another group was inoculated *intracardially* 1 week after the smooth infection. Two additional groups of animals were similarly inoculated 5 weeks after the primary S infection. A total of 80 guinea pigs was thus infected. In addition, 21 control animals were infected with S only, and 24 others received M cells only either subcutaneously or intracardially. Five animals from each group were sacrificed 1, 2, 4, and 6 weeks following the inoculation of M cells. From each of 3 of these 5 animals, 17 different tissues were cultured directly onto "2-1" agar plates at time of sacrifice, after thorough grinding with mortar and pestle. Four tissues only were cultured from the other 2 animals. After 4 days of incubation at 37 C, the 2-1 agar plates were inspected for brucella colonies, and whenever recoveries were obtained, the approximate number

of colonies and the percentage of S and M types among them were determined. The colony type identification was made with the help of a dissecting microscope and oblique lighting (Braun, 1946), and doubtful colonies were picked and restreaked on 2-1 agar plates in mixtures with the original S or M types. This checking procedure was occasionally necessary because it was found that in the presence of certain tissue, especially adrenal, testis, and mediastinal lymph node, the

RESULTS

Figure 1 summarizes the average proportion of S and M types in a number of tissues from animals that had been injected subcutaneously or intracardially with M cells 1 week after the S infection. It can be seen that the distribution of recoverable M cells differs significantly

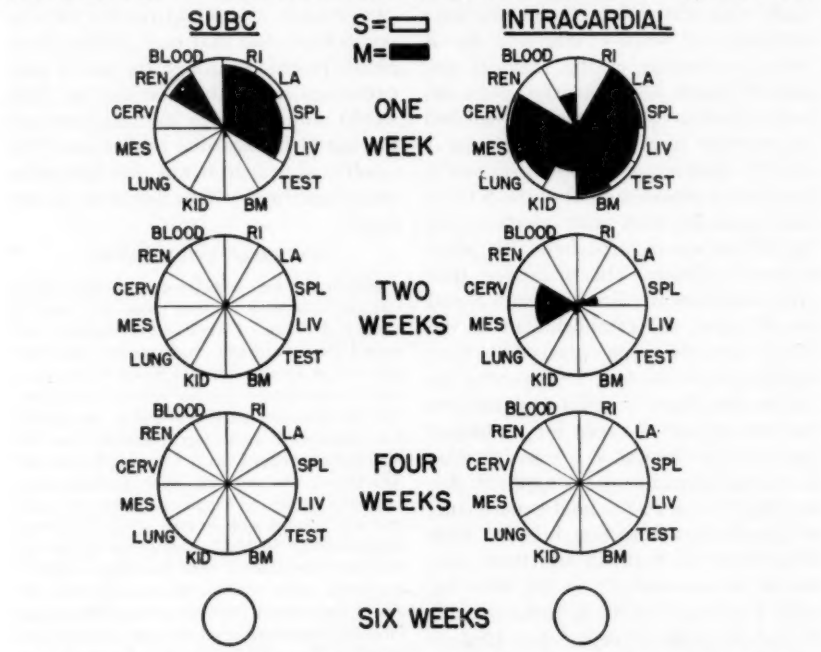

FIG. 1.—Average recovery of S and M cells from various tissues of guinea pigs infected subcutaneously or intracardially with M cells 1 week after subcutaneous S infection. Animals sacrificed 1, 2, 4, or 6 weeks after M infection. Black areas in sectors indicate the percentage of M, white areas the percentage of S cells recovered.

colony morphology of both S and M types was somewhat modified.

The mucoid variant used in these experiments originally was isolated during in vitro studies from the same smooth strain of *Br. suis* that has been used in the present guinea pig experiments. This M variant was recoverable from 2 out of 7 guinea pigs 4 weeks after subcutaneous infection with 940 cells per animal (Braun, 1950), but was recoverable from all animals infected with 2×10^9 cells.

depending on the route of inoculation. One week after the subcutaneous M injection, M cells were recoverable only from the right inguinal lymph node, left axillary lymph node, spleen, liver, renal or lumbar lymph node, and to some extent from the bone marrow. In contrast, all tissues from intracardially challenged animals sacrificed at the same time per-

mitted the recovery of a generally high percentage of M type cells. The relatively low percentage of M type cells recoverable from the right inguinal lymph node and the lumbar lymph node of these "intracardial" animals is noteworthy, especially since these two tissues yielded the highest recovery of M types in subcutaneously challenged animals. This may be attributable to some kind of blocking effect against the intracardially introduced M cells in the tissues closest to the site of the primary infection. In all instances the lumbar or renal lymph node yielded recoveries identical to those of the regional right inguinal lymph node, just as if there were a direct connection between those two lymph nodes. In general, a comparison between the two groups of animals indicates that a blocking effect by the lymphatic system follows the subcutaneous administration, whereas intracardial infection permits a general distribution of the variant type throughout the body of the host.

The next point of interest is the rapid disappearance of the M cells from most tissues within 4 weeks after infection. It can be seen that different tissues seem to possess a differential ability to rid themselves of the M type,—witness the greater persistence in the mesenteric and cervical lymph nodes. Although no detailed presentation of controls will be given, it was also observed that the M type cells disappeared much more rapidly from the tissues of animals previously infected with S type cells than from previously uninfected ani-

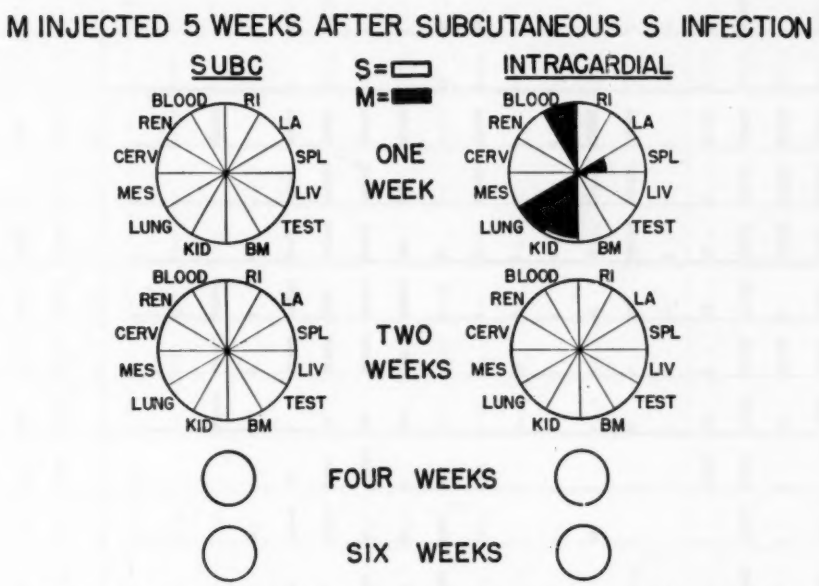

FIG. 2.—Average recovery of S and M cells from various tissues of guinea pigs infected subcutaneously or intracardially with M cells 5 weeks after subcutaneous S infection. Animals sacrificed 1, 2, 4, or 6 weeks after M infection. Black areas in sectors indicate the percentage of M, white areas the percentage of S cells recovered.

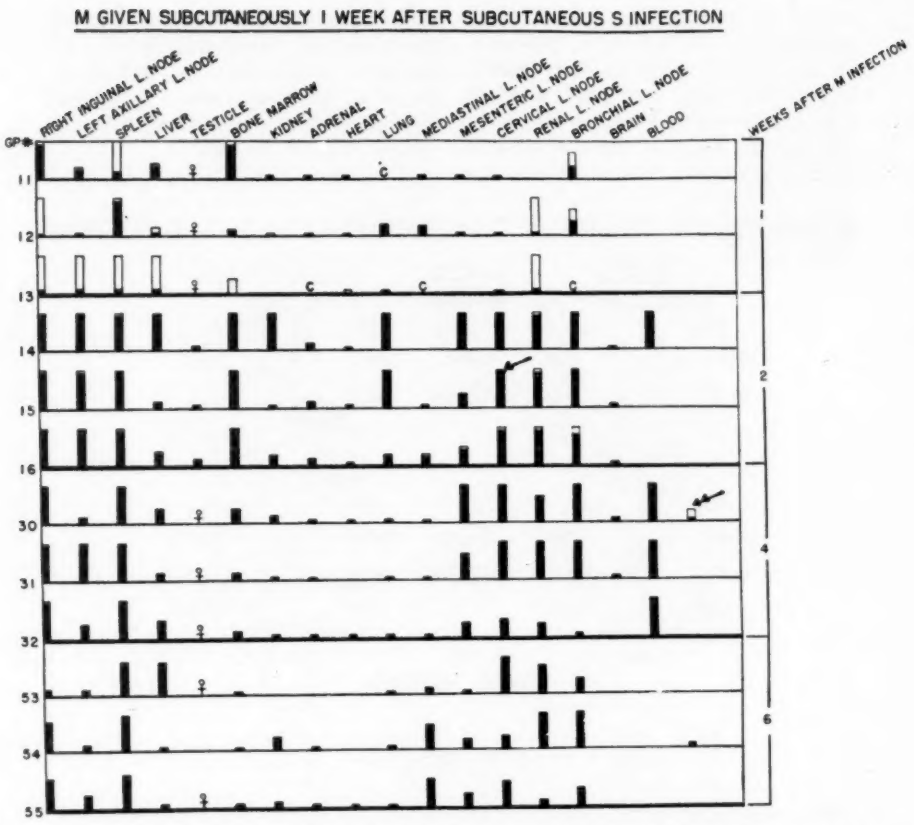

FIG. 3.—Recovery of S and M cells from individual guinea pigs infected with M subcutaneousy 1 week after subcutaneous S infection. Single arrow, guinea pig #15—2%I_s. Double arrow, guinea pig #30—abscess near RI.

FIG. 3–6.—The height of each column represents the relative amount of recovered cells. Solid portion of column = percentage of S; white portion of column = percentage of M.

mals. It can be seen that practically no M cells were isolated from animals sacrificed 2 weeks or more after secondary M infection, whereas in M-infected, previously unexposed animals the same M type cells were found to persist for more than 4 weeks.

Figure 2 shows that the disappearance of M types was even more rapid in animals challenged with M 5 weeks after S infection. No M types at all could be recovered from these tissues 1 week after subcutaneous administration of M cells to S-infected animals. As will be seen below, however, M types could actually be recovered from all these animals when abscesses near the site of infection were cultured. Although intracardial M challenge 1 week after S infection permitted good recovery of M types for at least 2 weeks, a similar challenge 5 weeks after infection with S yielded no

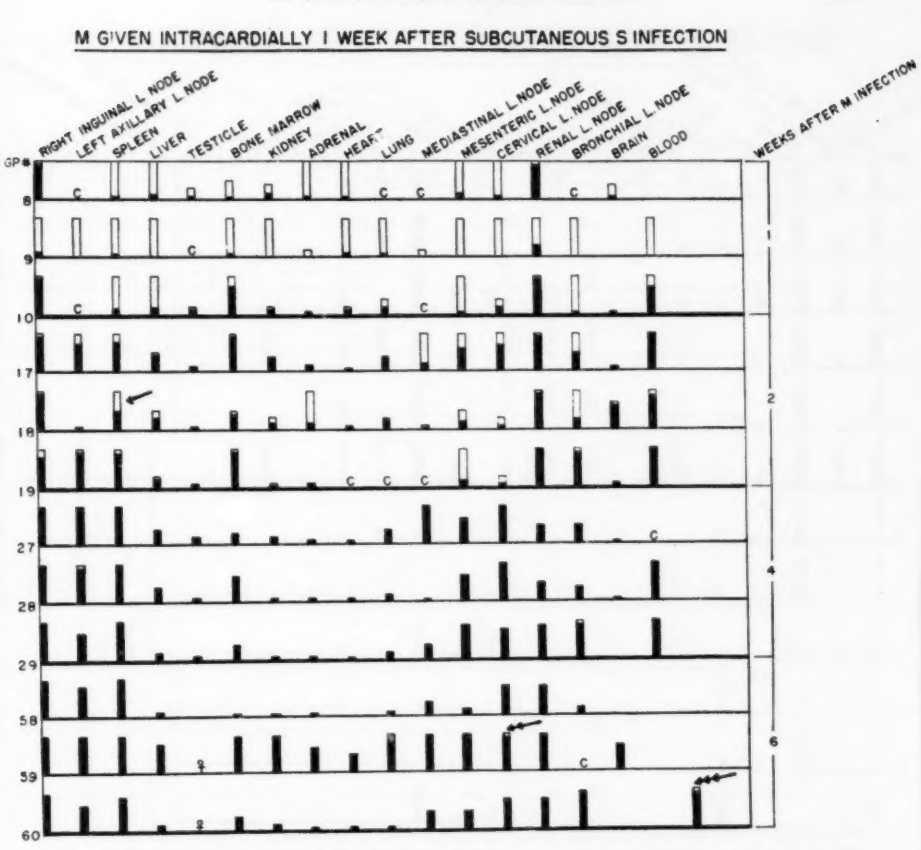

FIG. 4.—Recovery of S and M cells from individual guinea pigs infected with M intracardially 1 week after subcutaneous S infection. Single arrow, guinea pig #18—<0.01% I_2 in addition to M. Double arrow, guinea pig #59—1% I_2. Triple arrow, guinea pig #60—subcutaneous abscess.

M recoveries 2 weeks after reinfection.

In addition to these recoveries from various tissues, localized abscesses found in a number of animals were cultured. Most of these abscesses yielded M cells even when they came from animals that did not permit recovery of M cells from any of the other tissues. Figures 3 through 6 illustrate this. These figures show the more detailed results of the recovery from various tissues of individual animals. The height of each column indicates the relative amount of recovery; black areas represent S cells, white areas M cells. It was possible to isolate M cells from an abscess of animal #30 four weeks after the subcutaneous S infection (fig. 3). At that time no M cells could be isolated from any other tissue. In the following figure a similar example can be seen. Animal #60, which was sacrificed 6 weeks after intracardial M infection, contained an abscess in the submaxillary lymph node which yielded 5% M and 95% S cells. It will be noted in figure 5 that subcutaneous reinfec-

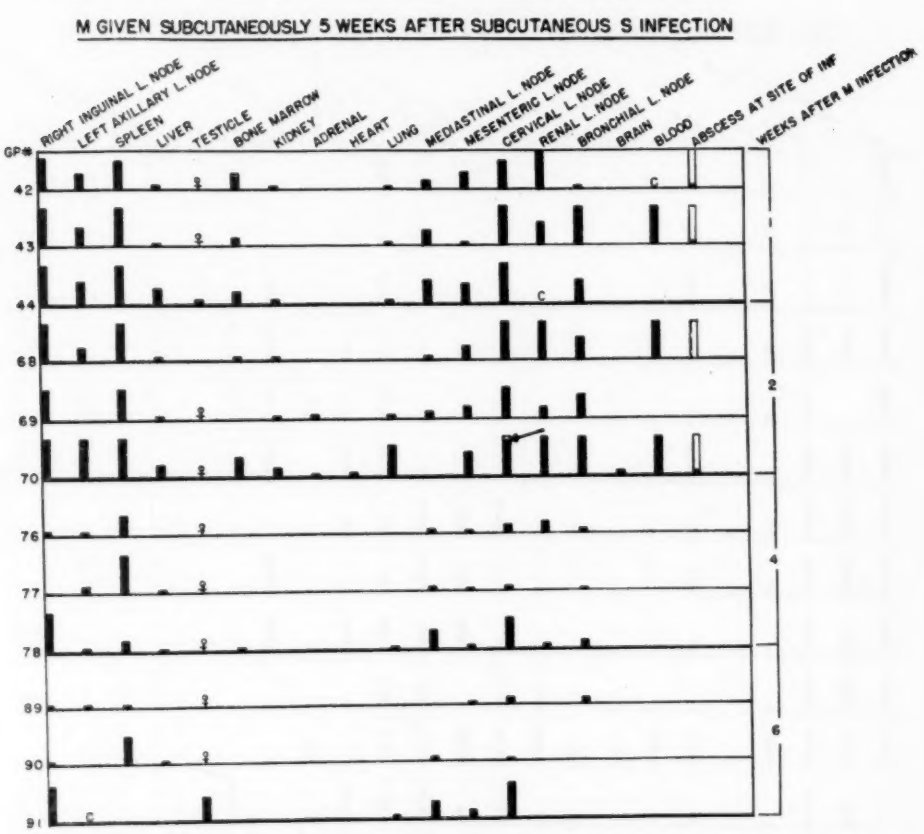

FIG. 5.—Recovery of S and M cells from individual guinea pigs infected with M subcutaneously 5 weeks after subcutaneous S infection. Single arrow, guinea pig #70—<1% I_3 in addition to M.

tion following 5 weeks after primary infection resulted in a number of abscesses at the site of reinfection. This simulates a Koch phenomenon in certain respects. M cells injected subcutaneously into animals 5 weeks after the S infection could not be recovered from any of the usual tissues cultured, but for 2 weeks following the M infection enormous numbers of M cells were found in the localized abscesses at the site of injection. Figure 6 furnishes 2 more examples of survival of less virulent nonsmooth types in localized lesions 4 and 6 weeks after intracardial M infection or 9 and 11 weeks after the S infection. One animal had a lung abscess containing 100% M cells; the other animal had a pericardial abscess containing 98% nonsmooth cells of a "rough" type plus 2% S cells. Inspection of figures 3 through 6 also reveals that the cervical and bronchial lymph nodes, as well as the spleen, appear to be preferential sites for long persistence of S cells.

DISCUSSION

In reviewing these data it may be

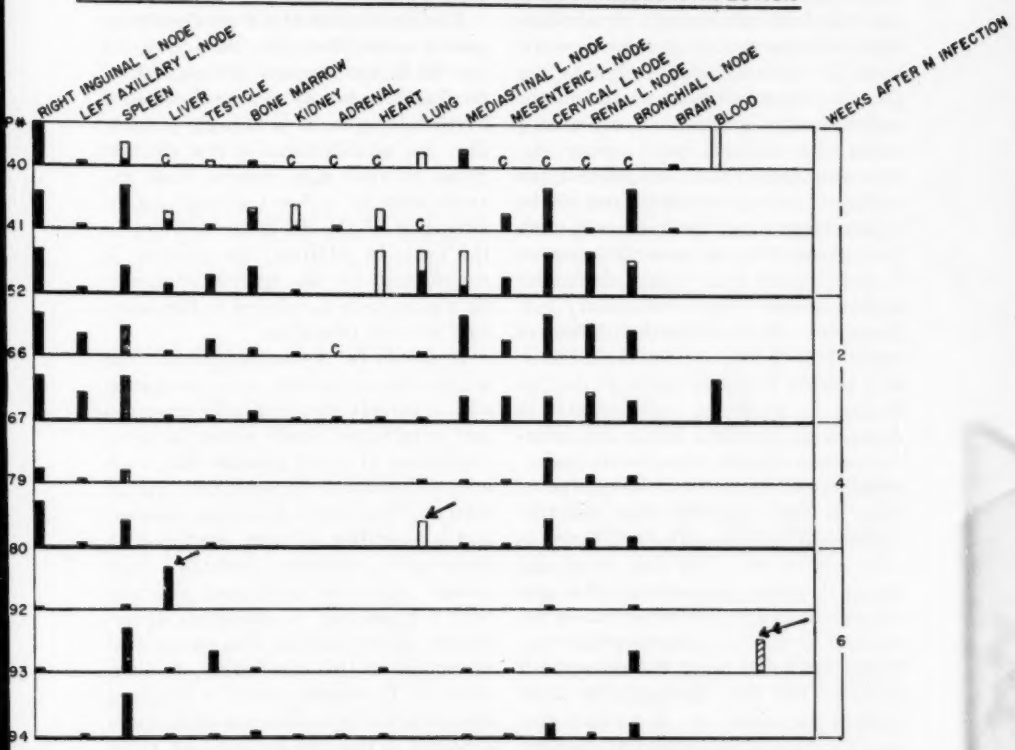

FIG. 6.—Recovery of S and M cells from individual guinea pigs infected with M intracardially 5 weeks after subcutaneous S infection. Single arrow, guinea pigs #80 and #92—abscess. Double arrow, guinea pig #93—I_4 in pericardial abscess.

concluded that, at least as far as the nonsmooth type used in these studies is concerned, there appears to be no opportunity for the less virulent type to establish itself generally in the host, although introduced at a time when the selective serum activity of the host might be expected to have changed. However, less virulent nonsmooth types, either introduced by injection or appearing spontaneously in vivo, seem to be able to persist in localized abscesses. Similar results have been reported in less extensive studies by Jones and Berman (1950). Further studies are required to confirm these isolated observations on the persistence of less virulent types in abscesses, a phenomenon that may be due merely to the protection from the normal clearing mechanisms furnished these cells by reason of their isolation. However, it should be pointed out that there is an alternate explanation that would be in good agreement with recent results obtained in test tube experiments. Goodlow et al (1950) reported that population changes in vitro are influenced by the accumulation of a toxic metabolite in originally smooth cultures. It was demonstrated

that brucella cells produce alanine as an end product of normal metabolism. When alanine accumulates to a certain level in the culture, it inhibits the growth of smooth types but permits the establishment of spontaneously arising nonsmooth mutants with greater alanine resistance. These nonsmooth types continue to produce alanine and will be replaced eventually by other, even more alanine-resistant, nonsmooth types, or in some strains by a highly alanine-resistant smooth type. Preliminary evidence also indicated that the addition of serum from a normal, susceptible host may modify the metabolism so that no alanine is produced and population changes are avoided. While this information was of great value for the understanding and control of variational processes, it also suggested that selective phenomena may be entirely different in vitro and in vivo. The data cited had shown that the accumulation of a specific metabolite plays a major role in the creation of specific environmental conditions that will favor the nonsmooth variants, but this accumulation took place in test tubes, i.e., in a closed system. The animal body probably does not represent such a closed system but rather an open system from which accumulating metabolites are removed continuously. Therefore, it would not be expected that conditions that affect population changes in vitro will be created to the same extent in most tissues of a host. However, a high concentration of metabolites could be expected in a closed environment as represented by abscesses.* Localized abscesses may, therefore, constitute the site at which the nonsmooth types find the environmental conditions that are necessary to endow them with a high selective value.

The significance of the previously reported serum effect, i.e., the activity of the SS factor in vitro (Braun, 1949), does not necessarily appear diminished by these results. It is entirely possible that the establishment of less virulent types in vivo may require both the availability of a closed system and an alteration of the SS factor activity in the host. In addition, the problem is complicated by the specific immunity that may have developed in the intervals between infections.

It should be re-emphasized that the studies here reported were conducted with a variant that originally appeared and established itself under in vitro conditions. It is still possible that variants with reduced virulence that arise in vivo might exhibit a different behavior, and a repetition of these studies with nonsmooth variants isolated from guinea pigs after prolonged infection with S types only is advisable. Nevertheless, reports such as that on survival of certain, in this case highly virulent, types of *Pasteurella pestis* in localized abscesses with simultaneous isolations of different, in this case less virulent, types from "open" tissues (Burgess, 1930), suggest that the results described above are not of isolated significance.

The differences observed in distribution of cells of the secondary infection, depending on the route of inoculation used, illustrate the danger of drawing general conclusions regarding the natural course of the disease from laboratory experiments employing the subcutaneous route of challenge only. The lymphatic blocking involved, including the reactions that are somewhat similar to a Koch phenomenon, may result in an atypical distribution of the parasites, which does not simulate cases where internal foci may serve as sources of infectious material. However, these block-

* Preliminary tests confirm that the concentration of free amino acids in abscesses differs quantitatively and qualitatively from that of normal tissues.

ing phenomena are of considerable interest in connection with immunological problems associated with brucellosis. For an excellent discussion of the latter considerations the reader is referred to the recent review by Elberg and Silverman (1950).

SUMMARY

Differences in colonial types, which usually are associated with differences in virulence, can provide simple markers for testing the fate of variants in vivo after simultaneous or consecutive inoculation of two or more variant types. Thus, in order to test the possibility that less virulent nonsmooth variants of *Brucella suis* may be capable of persisting in vivo following prolonged infections with highly virulent smooth (S) organisms, guinea pigs were inoculated subcutaneously or intracardially with massive doses of a mucoid (M) variant 1 week or 5 weeks after the primary S infection. Animals were sacrificed 1, 2, 4, and 6 weeks after the inoculation of M cells, and a variety of tissues were cultured. The distribution and persistence of M types in the various tissues differed significantly depending on the route of inoculation. A blocking effect and also reactions that resemble a Koch phenomenon in certain respects were produced by the lymphatic system after subcutaneous secondary infection. There appeared to be a differential ability of various tissues to rid themselves of the M type. With the exception of some localized abscesses, the less virulent M types did not persist in any tissue for prolonged periods. The details of these experiments have been presented, and their relationship to the results of in vitro studies as well as to problems of pathogenesis have been discussed.

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INFLUENCE OF SEX OF MICE ON HISTAMINE SENSITIVITY AND PROTECTION AGAINST *HEMOPHILUS PERTUSSIS*

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Recently Parfentjev and Goodline (1948) reported that pertussis vaccine markedly increases the sensitivity of mice to histamine. Since it has been suggested that an allergic reaction (Ann. Internal Med., 1950) is a possible cause of the few cases of encephalopathy which have been reported to occur after pertussis vaccination (Brody, 1947; Byers and Mall, 1948; Toomey, 1949; Globus and Kohn, 1949; Anderson and Morris, 1950), a series of studies of the histamine-sensitizing property of pertussis vaccines and *Hemophilus pertussis* was undertaken in the hope that it might provide an approach to the cause of this pathology. An encephalopathy also occurs sometimes during an infection of *H. pertussis* (see Litvak et al, 1948, for a recent review of the literature on cerebral complications in this disease). The results which will be presented in this and the following paper will be limited to those obtained with the use of mice. No attempt will be made to correlate them with encephalopathy in children.

In this paper there will be presented results which show that (1) the normal and the pertussis-vaccinated female mouse were more sensitive to histamine than the corresponding male mouse and that (2) pertussis vaccine afforded the female mouse better protection against intracerebral injection of *H. pertussis* than the male mouse.

METHODS

White mice from a closely inbred strain were used. They usually weighed around 16 g when tested for histamine sensitivity and around 12 to 14 g when vaccinated. Five days intervened between the injections of vaccine and histamine; both were given intraperitoneally. The histamine diphosphate dose was determined on a kilogram weight basis and given in 0.2 ml of distilled water. Food was removed 4 hours before injection of histamine. After injection of histamine the mice were observed for several hours and then kept overnight for a final check before discarding. The protective activity of the vaccines was determined by the intracerebral mouse-protection test as recommended by the National Institutes of Health (1948a). The 50% end point doses (LD_{50}, lethal dose of histamine; SD_{50}, sensitizing dose of vaccine) were calculated by the Wilson-Worcester method as adopted for the pertussis vaccine potency test (National Institutes of Health, 1948b).

EXPERIMENTAL

Reaction of the mouse to histamine.—Immediately after the injection of a lethal dose of histamine, the mouse appears excitable. Then it quickly becomes prostrate with hind legs extended backwards. The feet, ears and tail become blue; respiration is labored and becomes slower and slower until there are only gasps. There are frequent convulsions. Death usually occurs in less than 30 minutes—sometimes within 7 minutes but rarely after 60 minutes. Mayer and Brousseau (1946) reported similar reactions during anaphylactic shock of the mouse sensitized to horse serum. Usually the speed of death is directly re-

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lated to the degree of sensitization. At time of death the mouse is very flabby without muscle tonus. Death is associated with marked dyspnea. At autopsy, the diaphragm is found to be pushed up into the pleural cavity, the lungs are collapsed, and the right auricle and pulmonary arteries are markedly distended. The blood vessels in general are injected and petechial hemorrhages are seen in the subcutaneous tissue.

Comparison of the normal male and female mouse.—In the first experiment, which was done to determine the lethal dose of histamine for normal mice, the doses were varied by an increment of two. With 1250 mg/kg, no mice died; and with 2500 mg/kg, 5 of 10 male and 10 of 10 female mice died. In the next 2 experiments, which were done on successive days, an increment of 1.183 was used. The results are given in table 1. For the combined tests, a significantly different LD_{50} was obtained for the male and female mouse; they were 2259 and 1932 mg/kg, respectively. The ratio of male to female is 1:0.856 or of female to male, 1:1.17. Further, when the 3 tests were analyzed by using the difference in response of the male and female at each dose employed, a value of $t = 3.443$ was obtained. This falls between the 98 and 99% confidence intervals, 3.143 and 3.707, respectively, for the number of samples involved.

TABLE 1.—*Histamine sensitivity of normal male and female mice.*

Sex	Histamine diphosphate	Deaths/number injected			A and B
		A	B	A and B	LD_{50} of histamine* (2 S.E. limits)†
	mg/kg				mg/kg
Male	1250	0/10			
	1750	1/10	0/10	1/20	2259
	2070	2/10	7/10	9/20	(2086 and
	2450	6/10	6/10	12/20	2446)
Female	1250	0/10			
	1750	1/10	4/10	5/20	1932
	2070	7/10	7/10	14/20	(1811 and
	2450	8/10	10/10	18/20	2061)

LD_{50} difference between female and male = log 0.0679 ± log 0.0446 (2 S.E.).
* The LD_{50} ratio of female mouse to male mouse is 1:1.17.
† Two standard error limits or 95% fiducial limits.

TABLE 2.—*Histamine sensitivity of vaccinated male and female mice: variation in amount of histamine.*

Sex	Pertussis vaccine	Histamine diphosphate	Deaths/number injected	LD_{50} of histamine* (2 S.E. limits)
	million	mg/kg		mg/kg
Male	2500	12.5	0/10	46.5
	2500	25.0	0/10	(36.7 and 59.4)
	2500	50.0	6/10	
Female	2500	12.5	0/10	25
	2500	25.0	7/10	(18.5 and 34)
	2500	50.0	8/10	

LD_{50} difference between vaccinated female and male mouse = log 0.2710 ± log 0.1686 (2 S.E.).
* The LD_{50} ratio of female mouse to male mouse is 1:1.86.

Comparison of vaccinated male and female mice.—The sensitivity of the vaccinated male and female mice was compared by varying the doses of both the histamine and the pertussis vaccine. In table 2 are given the results of an experiment in which the mice were given a constant dose of vaccine and varying amounts of histamine. As with the normal mouse, the female mouse was significantly more sensitive than the male mouse, and in this experiment the difference was greater than was found in the normal mice. It should be noted that the mice vaccinated with 2500 million bacteria were approximately 50 times or more sensitive to histamine than the normal mice.

In table 3 are given the results of 3 experiments in which the dose of vaccine was varied and the amount of histamine was kept constant. A larger increment between doses was used than with histamine. Therefore the probability of error was greater. In each test the SD_{50} of vaccine was less for the female than for the male, but the difference within a single test was not statistically significant. The tests could not be combined to obtain a SD_{50} because the doses of vaccine and histamine were not the same in all. However, when they were analyzed for t, a value exceeding the 95% confidence interval was obtained.

Survival of male and female mice in

TABLE 3.—*Histamine sensitivity of vaccinated male and female mice: variation in amount of vaccine.*

Exp. no.	Pertussis vaccine	Histamine diphosphate	Deaths/number injected		Analysis of combined tests
			Male	Female	
	million	mg/kg			t
1	5000	60	7/10	10/10	
	1250		5/10	6/10	2.7137
	312		1/10	3/10	$t_{0.05}=2.262$
2	5000	60	8/10	10/10	
	1250		8/10	9/10	
	312		2/10	2/10	
3	5000	50	7/10	8/10	
	2000		6/10	8/10	
	800		4/10	2/10	
	320		0/10	2/10	

protective tests of pertussis vaccines.—After it was observed that there was a sexual difference in the response of mice to histamine, a record was kept of the number of male and female mice that survived in 8 consecutive protective experiments of pertussis vaccines. In each 7 to 9 vaccines were included. Three varying doses of vaccines were used for each vaccine. With each dose 9 male and 8 female mice were vaccinated. After the intercerebral injection of culture, if there was no traumatic death, the weakest looking male mouse was discarded. This was done to assure 16 mice per dose of vaccine. The results are summarized in table 4. It may be seen in each experiment that the percentage of survivals was lower for the male mice than for the female mice. The over-all ratio is 1:1.55. If it is assumed that there was no difference in the response of the male and female mouse, the probability is that one-half of the survivals would be males. In each of 8 consecutive tests more female than male mice survived. With this number the probability is 1:256 that an equal response would be obtained with mice of the opposite sex in an experiment of 8 vaccines.

Since the totals given in the table do not take into account the dosages of vaccine, the influence of dosage might be questioned. Three doses containing 1500, 300 and 60 million bacteria had been used. Accumulations were made of the mice at the respective doses only when equal numbers of mice of each sex had been injected and when all mice had not survived. The results obtained were 195 and 273, 80 and 129, and 25 and 46 male and female mice, at the respective doses. The ratios, which are 1:1.40, 1:1.61 and 1:1.84, suggest that the dosage may be influential. Nevertheless, it is obvious that, irrespective of the dosage of vaccine, fewer male than female mice survived.

It is of particular interest that the ratio of the LD_{50} of histamine for the vaccinated female and male mice is not very different from the ratio of the male and female survivals in the protective tests. The former was 1:1.86 and the latter, for all mice, was 1:1.55; but for the smallest dose of vaccine it was 1:1.84. A comparison of the histamine-sensitizing and the protective properties of pertussis vaccines will be presented in the following paper.

DISCUSSION

In the work presented in this paper, it

TABLE 4.—*Survival of male and female mice in protective tests of pertussis vaccines.*

Exp. no.	Number of vaccines	Survivals/number injected		Percentage of survivals		Ratio of survivals, male:female
		Male	Female	Male	Female	
176	9	40/124	54/114	32.3	47.4	1:1.47
177	8	51/147	56/137	34.7	40.9	1:1.18
178	8	34/104	44/107	32.7	41.1	1:1.26
179	9	54/165	85/171	32.7	49.7	1:1.52
180	8	44/178	93/174	24.7	53.4	1:2.16
181	8	56/184	71/183	30.4	38.8	1:1.28
182	7	45/135	87/137	33.3	63.5	1:1.91
183	8	71/178	122/189	39.9	64.6	1:1.62
Totals	65	395/1215	612/1212	32.5	50.5	1:1.55

No mice were included at a given dose if all mice either survived or died.

has been shown that there was a significant difference in the reaction of the male and female mouse to histamine, both in the normal and the pertussis-vaccinated mouse, and also in the protection of the vaccinated mouse against *H. pertussis*. Differences in reaction in animals of the opposite sex is not a new observation, but too little attention has been given to it in experimental tests. The findings reported here emphasize the need of taking into account the variability of the response of the mouse due to sex, both in experimental work and in the standardization or evaluation of biological and chemical substances. An analysis of a large number of protective tests done to evaluate the potency of pertussis vaccines showed that a significantly greater number of female than male mice survived. The ratio of male to female was 1:1.55. This difference could materially affect the results of the determination of the potency of a vaccine, if the variable was not controlled. The use of mice of one sex would be highly desirable.

It is suggested from the results that pertussis vaccine may enhance the difference in the reaction found in the normal male and female mouse. The difference was greater in the histamine sensitivity of pertussis-vaccinated mice than in normal ones. With vaccinated mice, a difference of the same magnitude in sensitization and protection was observed. Female mice were more sensitive and were better protected with vaccine than were male mice. In connection with the influence of sex in response to infection, it is of interest to recall that in children mortality caused by pertussis is higher in the female than in the male child (U. S. Pub. Health Service, 1944–1947).

SUMMARY

In this study of the influence of sex of mice on histamine sensitivity and protection against *Hemophilus pertussis*, the following observations were made: (1) In normal mice, the female mouse was more susceptible to histamine than the male mouse. (2) In pertussis-vaccinated mice, the histamine sensitivity of which had been increased manyfold, the female mouse was also more sensitive to histamine than the male mouse. The difference appears to be greater than in the normal mouse. (3) In protective tests, more female than male mice survived; the difference was found to be of the same magnitude as between the histamine sensitivity of the pertussis-vaccinated male and female mice.

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COMPARISON OF THE HISTAMINE-SENSITIZING PROPERTY WITH THE PROTECTIVE ACTIVITY OF PERTUSSIS VACCINES FOR MICE

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This paper is the second of a study of the histamine-sensitizing properties of pertussis vaccine and *Hemophilus pertussis*. In the first paper, which dealt with influence of sex, it was reported that (1) the normal and the pertussis-vaccinated female mouse were more sensitive to histamine than the corresponding male mouse and (2) in protective tests more female mice than male mice survived (Pittman, 1951). Parfentjev and Goodline (1948) first showed that pertussis vaccine markedly increases the sensitivity of mice to histamine diphosphate.

In the present paper, it will be shown that the usual fluid pertussis vaccines which induced the highest degree of histamine sensitization in mice usually afforded mice the best protection against cerebral infection, that a detoxified vaccine sensitized the animal better than a toxic vaccine and that the addition of alum to the vaccine somewhat altered the sensitization response.

METHODS

In general, the methods were the same as were used in the work reported in the previous paper (Pittman, 1951). In the histamine tests only female mice were used. In the protective tests equal numbers of male and female mice were employed. The sensitizing action of a vaccine was determined either by giving a constant dose of vaccine (1500 million bacteria) followed by varying amounts of histamine, or conversely, by varying amounts of vaccine (5000, 1250 and 312 million bacteria) and a constant amount of histamine diphosphate (50 mg/kg). Both methods gave a regression curve. In the former the deviation from the curve was somewhat less than the latter. In general, however, we found it more practical to vary the amount of vaccine and keep the dose of histamine constant. Five days intervened between the injection of vaccine and histamine unless stated otherwise. All injections were made intraperitoneally.

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EXPERIMENTAL

Protective and histamine-sensitizing action of commercial vaccines.—In table 1 is given a comparison of the ED_{50} and the SD_{50} of 6 commercial plain pertussis vaccines. The vaccines were tested at the same time for protection and likewise for sensitization. The protective doses of the vaccines are arranged from low to high values. These fall into 4 groups. The sensitizing doses of the vaccines, with the exception of no. 2, fall into the same 4 groups. The ratio between the ED_{50} and the SD_{50} of each of these 5 vaccines is strikingly close. It is definite that the correlation obtained here in 4 groups is not due to chance alone. A number of vaccines have been tested, and in general it has been observed that vaccines with high protective value induced high sensitivity to histamine and, conversely, with low sensitivity there was low protection. Two circumstances in which correlation did not occur are given below.

Influence of 37 C on protective and sen-

TABLE 1.—*Protective and histamine sensitizing action of 6 commercial vaccines.*

Vaccine no.	Culture—100,000 bacteria		Histamine—50 mg/kg		Ratio of ED_{50}:SD_{50}
	ED_{50}	1 S.E. limits	SD_{50}	1 S.E. limits	
	million	million	million	million	
1	376	278 and 504	884	580 and 1350	1:2.35
2	376	278 and 504	1920	1432 and 2580	1:5.13
3	609	426 and 871	1458	1076 and 1970	1:2.39
4	840	605 and 1176	2000	1420 and 2840	1:2.38
5	840	605 and 1176	2300	1640 and 3225	1:2.74
6	2182	1091 and 4342	4600	3550 and 5980	1:2.11

sitizing properties of a vaccine.—A portion of a concentrated vaccine which was free of dermanecrotic toxicity was diluted and divided into three parts. To 2 merthiolate was added, and to 1 phenol. One portion containing merthiolate was kept at 6 C and the other two at 37 C for 30 days. Then the protective and sensitizing properties were compared. The results are given in table 2. Heating reduced both properties but not in the same ratio. It is apparent that the sensitizing property was affected more than the protective property.

Comparison of a toxic and a detoxified vaccine.—*H. pertussis* contains an endotoxin which induces dermanecrosis in rabbits and death in mice. This property is destroyed at 56 C in less than 30 minutes (Lawson, 1933; Katsampes et al, 1942). To observe whether this property might play a role in the sensitization of mice to histamine, a fresh vaccine, no. 1 (5), was prepared. One portion was detoxified by heating at 56 C for 30 minutes, preserved with 0.01% merthiolate and then stored at 6 C. The other portion was kept at 6 C; before storage merthiolate was added to a concentration of 0.02% to kill the bacteria and then the vaccine was diluted to give a final concentration of 0.01%. This portion was capable of inducing dermanecrosis in rabbits and killing mice. The 2 portions were tested for protective and histamine-sensitizing activity when the vaccine was 5 and 6 days old, respectively. The results are given in table 3. The sensitizing dose of the toxic vaccine was about 8 times that of the detoxified vaccine. In contrast, the 50% protective doses of the 2 vaccines were so close that they fell within the limits of probable variability. It should be noted, however, that at the largest dose fewer mice were afforded protection by the toxic vaccine. At the 2 lower doses, comparable protection was obtained with each portion. A comparison of the values of α indicates that the responses to the 2 portions were different, i.e., the curves are not parallel. Therefore the relationship at the 50% point would not hold at all points on their curves. In a former paper Pittman and Liebermann (1948) called attention to the lack of parallelism in protection afforded by a toxic and a detoxified vaccine. Although there is lack of parallelism in protective

TABLE 2.—*Decrease of protective and sensitizing activities of a vaccine by heating at 37 C.*

Treatment of vaccine NIH no. 4	Preservative	ED_{50} (1 S.E. limits)	SD_{50} (1 S.E. limits)
Kept at 6 C	Merthiolate, 0.01%	million 519 389 and 695	million 1090 843 and 1403
30 days at 37 C	Merthiolate, 0.01%	1297 1012 and 1660	4600 2965 and 7125
30 days at 37 C	Phenol, 0.5%	1500 1140 and 1995	Only 1 death

TABLE 3.—*Sensitization and protection induced by a toxic and detoxified vaccine.*

Treatment of vaccine no. 1(5)	Sensitization†			Protection‡		
	Dose of vaccine	Deaths/number injected	SD_{50} (2 S.E. limits)	Dose of vaccine	Survivals/number injected	ED_{50} (2 S.E. limits)
	million		million	million		million
6 C (toxic)	5000	4/6*	2300	1500	11/16	382
	1250	4/10	1310 and 10,390	300	10/16	184 and 790
	312	0/10		60	1/16	α
	78	0/10				1.06 ± 0.32
56 C for 30 min., then 6 C	5000	10/10	312	1500	15/16	247
	1250	9/10	154 and 634	300	11/16	182 and 335
	312	4/10		60	0/16	α
	78	2/10				2.52 ± 0.74

α = value of the parameter of the slope of the curve.
* Four mice died from toxicity before injection of histamin..
† Vaccine 6 days old.
‡ Vaccine 5 days old.

response, it appears to be definite that the correlation between protection and sensitization observed with the aged vaccines above did not hold with a toxic and a detoxified vaccine.

Alum-precipitated vaccines.—An attempt has been made to determine the influence of alum on the histamine-sensitizing activity of pertussis vaccines. The activity of a number of vaccines with and without alum have been compared. The results have not been clear-cut, but it is apparent that the presence of alum does cause some alteration in the response. The results obtained with 3 vaccines are given in table 4. With the first 2, the SD_{50} of the portion containing alum was larger at 5 days but smaller at 10 days than those for the corresponding plain vaccine. With the 3rd vaccine, the LD_{50} of histamine was less both at 5 and 10 days for the mice that received the vaccine containing alum. In all cases, a marked drop in sensitization had occurred by the end of 15 days. It is indicated that the presence of alum may delay or prolong the absorption of the sensitizing factor, thereby causing a delay in the peak of sensitization and possibly increasing the degree of sensitization. In any case, high sensitivity does not persist for a long period.

The 50% protective doses of 2 vaccines are also given in table 4. The same doses were obtained with the 2 portions of 1 vaccine, while the dose of the alum-containing portion of the other was about one-half of that of the plain por-

TABLE 4.—*Influence of alum precipitation of vaccine on sensitization.*

Vaccine no.	Sensitization			Protection
	5 days	10 days	15 days	ED_{50} (1 S.E. limits)
	SD_{50} (1 S.E. limits) of vaccine			
	million			million
No. 4 plain	680	8350		868‡
	345 and 1335	3565 and 12,370		660 and 1369
No. 4 alum*	1886	3680	4000	840‡
	1118 and 3120	2195 and 6180	3010 and 5320	664 and 1058
No. 76 plain	1720	1720	5510	1036§
	1210 and 2444	1210 and 2444	3650 and 8330	839 and 1285
No. 76 alum†	2270	1250	4410	434§
	1348 and 3830	970 and 1612	3220 and 6030	230 and 816
	LD_{50} (2 S.E. limits) of histamine			
	mg/kg			
No. 857 plain	37.5	40.6	largest dose: 75 mg/kg	
	26 and 53	30 and 55	4D/10	
No. 857 alum	16.1	21.3	84.6	
(1.0 mg per dose)	4.5 and 57	10 and 45	52 and 138	

* Supernate was discarded after precipitation.
† Contained diphtheria and tetanus toxoids.
‡§ Tested at same time, respectively.

tion. This lack of consistent influence of alum on the protective response in mice is frequently observed in the routine testing of pertussis vaccines.

DISCUSSION

In the work presented it has been shown that with some pertussis vaccines there was a correlation between the protective and the histamine-sensitizing activities. Vaccines that afforded good protection to mice induced high sensitivity and, conversely, with poor protection there was low sensitivity. Evidence was presented, however, that indicates that the 2 factors are not interrelated. By heating at 37 C for 30 days, especially in the presence of phenol, the loss in ability to induce sensitivity to histamine was much greater than the protective activity; and the sensitizing dose of a toxic vaccine was 8 times greater than that of the same vaccine which had been detoxified by a short period of heating at 56 C.

Although it appears that the histamine-sensitizing and protective activities are not interrelated, it is probable that the reaction of one may affect indirectly the other. The peak of sensitivity and protective response do not coincide. Parfentjev (1950) found that the peak of histamine sensitivity induced by a fluid vaccine is around 5 days. This we have confirmed. During the experimental work (unpublished) on the mouse test for the minimum requirements: pertussis vaccine (1948), it was found that better protection was obtained after a 10-day than a 5- or 7-day rest period. Furthermore, with vaccines containing alum a rest period of at least 14 days was usually indicated. In comparison with the corresponding doses of the plain vaccine, the tendency was to obtain less protection with the largest dose and better protection with the smallest. The resulting curve was much flatter. The fact that the small dose of alum vaccine affords equal and sometimes better protection suggests that some factor other than absorption may be influential in altering the protective response.

At present there is no apparent explanation for the physiological reaction that is manifected by the increased sensitivity to histamine. Some years ago Eldering (1941) showed that a nontoxic carbohydrate from *H. pertussis* induced in mice a sensitivity to the living bacteria. Parfentjev (1950) found that pertussis-vaccinated mice were also sensitive to a pertussis "bacterial protein (antigen)." The peak of this antigenic sensitivity was on the 11th or 12th day, in contrast to the histamine sensitivity around the 5th day. It appears that sensitivity to histamine is not due to a specific antigenic sensitivity. Two papers have reported blood sugar changes following injection of *H. pertussis* fractions. One was by Evans (1943) and the other by Parfentjev and Schleyer (1949). In the latter, it was reported that a hypoglycemia present in the sensitized mouse was increased by injection of histamine.

The histamine sensitivity of mice during a respiratory infection of pertussis will be reported in a subsequent paper. What bearing the observations made with the use of mice may have on the behavior of human beings towards pertussis vaccine or infection must await further investigation.

SUMMARY

The histamine-sensitizing and the protective properties of pertussis vaccine have been compared. With ordinary fluid vaccines there was usually a close correlation between the two properties. By prolonged heating the sensitizing property was reduced relatively more than the protective activity. A toxic

vaccine, which was capable of inducing dermanecrosis, induced much less sensitivity than a portion of the same vaccine after destruction of the toxin by a short period of heating. The 50% protective doses of the two portions were not significantly different. The presence of alum in the vaccine somewhat altered the sensitivity response b causing a slight delay in the peak of sensitization, a slight prolongation and at times a higher degree of sensitivity. The influence of alum on the mouse-protective action of pertussis vaccine was discussed.

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VOLUME 89 NOVEMBER-DECEMBER 1951 NUMBER 3

The Journal of Infectious Diseases

TABLE OF CONTENTS

PAGE

Norman B. McCullough and C. Wesley Eisele. Experimental human salmonellosis. III. Pathogenicity of strains of *Salmonella newport*, *Salmonella derby*, and *Salmonella bareilly* obtained from spray-dried whole egg 209

Lois M. Jones and David T. Berman. The pathogenicity of mucoid variants of brucellae for guinea pigs 214

Samuel Spector and Herbert D. Mercer. The effect of pituitary adrenocorticotrophic hormone on experimental tetanus in mice 224

Eric L. Nelson and M. J. Pickett. The recovery of L forms of brucella and their relation to brucella phage 226

William Burrows. Kinetic aspects of hemolysis by the α-toxin of *Clostridium welchii* and its inhibition by antitoxin 233

Florence B. Seibert, Crystal Crumb and Emma H. Dufour. Antigenic differences in two tuberculin protein fractions 252

Norman B. McCullough and C. Wesley Eisele. Experimental human salmonellosis. IV. Pathogenicity of strains of *Salmonella pullorum* obtained from spray-dried whole egg 259

Norman B. McCullough and Grace A. Beal. Growth and manometric studies on carbohydrate utilization of *Brucella* 266

Abraham I. Braude and Wesley W. Spink. Studies in the pathology and pathogenesis of experimental brucellosis. III. Investigations pertaining to the function of the spleen 272

Robert S. Bowles and E. Slater Weyer. Studies on the mechanism of antagonism by *Aerobacter aerogenes* 277

Luis W. Gonzalez and Enrique Koffinch. Studies in bacillary dysentery. III. Mobilization of specific antibody into the intestine of the rabbit as a factor in its hemol-gous living dysentery bacilli 1

William Braude, Stephen M. Gorelick, Harry Kraft and Dorothy D. Mead. Studies on the in vivo fate of brucella variants with different virulence 286

Margaret Pittman. Influence of sex of mice on histamine sensitivity and immunity against *Hemophilus pertussis* 295

Margaret Pittman. Comparison of the histamine-sensitizing property with the protective activity of pertussis vaccines for mice 300